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The use of monoclonal and polyclonal antibodies to identify Escherichia coli

Charles William Kaspar

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The use of monoclonal and polyclonal antibodies to identify *Escherichia coli*

Kaspar, Charles William, Ph.D.

Iowa State University, 1986
The use of monoclonal and polyclonal antibodies to identify Escherichia coli

by

Charles William Kaspar

A Dissertation Submitted to the Graduate Faculty in Partial Fulfillment of the Requirements for the Degree of

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ABSTRACT

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GENERAL INTRODUCTION

Human intestinal pathogens are usually found in food and water in low numbers which makes detection difficult. Therefore, readily identified natural inhabitants of the intestinal tracts of animals are used to signal the presence of fecal contamination and the possible presence of pathogens. There has been much debate about which index organism(s) provides the best signal for the presence of a health hazard. Currently, coliforms, fecal coliforms, and *Escherichia coli* are used as index organisms. Because members of the coliform and fecal coliform groups are normal inhabitants of some environments, their presence in a sample of water or food may not always be of sanitary significance. *E. coli* is restricted to the intestinal tract of warm-blooded animals and is usually present in numbers greater than other coliforms and pathogens. Because of its specificity, *E. coli* is often preferred as an indicator of fecal pollution. However, conventional methods for the isolation and identification of *E. coli* among other coliforms require considerable time and expense. Many improvements in the detection of *E. coli* have been made. Nevertheless, additional methods that are more rapid, and methods which can complement existing methods, are needed.

Enzyme immunoassay and coagglutination tests have provided a rapid means of identifying the presence of bacteria. Both procedures require specific antibody, which, in most instances, is directed to cell-surface antigens present on the organism being identified. Identification of
E. coli by these methods is difficult because of the numerous serogroups and the presence of cross-reacting antigens. However, a fluorescent antibody test has been described for the detection of E. coli; cross-reacting antibodies were removed by adsorption before the antiserum could be used in this test. Major problems with the fluorescent antibody procedure are that expensive equipment is required to automate the procedure, it is tedious to perform, and the results are often subjective.

The present study was undertaken to determine if polyclonal or monoclonal antibodies to heat-treated E. coli or E. coli enzymes could be used in an enzyme immunoassay or coagglutination test for detection of E. coli in water and food.

Dissertation Format

This dissertation is presented in the alternate format and includes two manuscripts to be submitted for publication in Applied and Environmental Microbiology. The format used is that of the American Society of Microbiology. A literature review precedes the first manuscript. At the end of each manuscript, a separate literature cited section has been included. A general summary and discussion follow the second manuscript.

Charles W. Kaspar was the principal investigator in both of these studies.
LITERATURE REVIEW

Marker Organisms

History

The use of bacteria as indicators of water quality probably began in 1880 when Von Fritsch described *Klebsiella pneumoniae* and *Klebsiella rhinoscleromatis* as microorganisms characteristically found in human feces (cited in Geldreich, 1978). Soon thereafter, Escherich reported that *Bacillus coli*, later to become *Escherichia coli*, was one of the predominant organisms found in human feces (Escherich, 1887). Since *B. coli* was a prominent member of the gut flora, it should be associated with any intestinal pathogens present. Based on this concept, Schardinger (1892) proposed the use of *B. coli* to signal the presence of fecal contamination. Thus, testing for specific pathogens, such as *Salmonella*, was replaced with tests for *B. coli* (*E. coli*) which were more easily recovered and identified than pathogens. Later, it became apparent that *E. coli* belonged to a group of morphologically and physiologically similar organisms that inhabit the intestines of warm-blooded animals. This group of bacteria became known as the coliforms. Coliforms replaced *E. coli* as the indicator standard in 1914 (Levine, 1961), irrespective of the fact that some coliforms had been shown to be nonfecal in origin (Metcalf, 1905). To increase the fecal specificity of the coliform group, Eijkman (1904) devised a test whereby fecal coliforms were distinguished from nonfecal coliforms by their ability to produce gas from glucose when incubated at 46°C. Currently, coliforms, fecal coliforms,
and E. coli are all used as marker organisms (APHA, 1984). More complete reviews on bacteria used as markers of fecal pollution have been published (Hoadley and Dutka, 1977; Mossel, 1978, 1982).

To avoid confusion in the terminology used to describe marker organisms, Ingram (cited in Mossel, 1978) suggested that index organisms should be used to refer to bacteria whose presence signals the presence of pathogenic organisms. The term indicator organisms should be used to refer to the general bacteriological state of a sample. This terminology will be used throughout the remainder of this discussion.

An ideal index organism must conform to several criteria (Dutka, 1973). First, the organism must be associated with the source of the pathogen. Secondly, the organism should be present in greater numbers than the pathogen. Thirdly, the organism should not proliferate in the environment. Fourthly, the index organism should be as resistant or more resistant than pathogens to environmental stresses. Finally, the index organism should be easily recovered and identified. To date, there are no index organisms which satisfy all these criteria.

Coliforms

Coliforms by definition are aerobic or facultatively anaerobic, non-sporeforming, gram-negative rods, that ferment lactose to acid and gas within 48 h at 35°C (APHA, 1985b). Since this definition is somewhat vague, a relatively diverse spectrum of bacteria is encompassed. Representatives of some species of bacteria which fall into this category are widely distributed throughout the environment and have little
significance with regard to health hazards. For example, *Aeromonas* sp. are facultatively anaerobic, gram-negative rods, frequently found in water. Some strains of *Aeromonas* are capable of fermenting lactose, so they may be falsely identified as *E. coli* (Austin et al., 1981; Neilson 1978). *Enterobacter aerogenes* and *Enterobacter cloacae* have been found on various types of vegetation (Geldreich, 1978) and soil (Duncan and Razzell, 1972). They have also been reported to multiply on leather washers, wood, swimming pool ropes, and bacterial film found in pipes (APHA, 1985b). *Klebsiella* sp. have been isolated from undisturbed soils and from tree bark (Dutka, 1979). These environmental isolates of *Klebsiella* cannot be distinguished from strains of human origin (Hendricks, 1978). Additional problems have been identified in waters containing high concentrations of carbohydrates and waters receiving certain types of industrial effluents. Dutka (1979) reported that coliforms from soil and bark multiplied in paper mill effluents. Discharge of these effluents led to increased coliform counts in the receiving waters. The increases in coliform numbers were caused primarily by the presence of *Klebsiella* spp., which are of little sanitary significance. Thus, in certain samples, elevated coliform counts do not necessarily represent a health hazard (Caplenas and Kanarek, 1984; Weiss et al., 1983).

In addition to the ubiquitous nature of coliforms, their presence correlates poorly with the presence of pathogens in food and water (Andrews et al., 1975; Gallagher and Spino, 1968; Miskimin et al., 1976). A number of outbreaks of gastroenteritis have involved water which by
coliform standards should have been safe (Boring et al., 1971; Dutka, 1973; Gallagher and Spino, 1968). Coliforms have also been reported as a poor index of enteric viruses (Berg, 1978).

**Fecal coliforms**

To alleviate the problem caused by environmental strains of coliforms, Eijkman (1904) divided the coliforms into fecal and nonfecal coliforms based on the ability of fecal coliforms to produce gas from glucose at 46°C. The test was later modified by substituting lactose for glucose and incubating at 44.5°C rather than 46°C (Perry and Hajna, 1944). Fecal coliforms have all the characteristics of coliforms plus the ability to ferment lactose with gas production in 24 h at 44.5°C (APHA, 1985b). There are several advantages of using fecal coliforms rather than total coliforms as index organisms. Fecal coliforms are more specific to fecal origin; they are found in relatively high numbers in feces; and they do not proliferate in the environment (Geldreich, 1967).

One of the criticisms of fecal coliforms as index organisms concerns their resistance to environmental stress. Reports are divided on the relative resistances of fecal coliforms versus pathogens (Andrews et al., 1975; Gallagher and Spino, 1968; McFeters et al., 1974; Miskimin et al., 1976; Xu et al., 1982). Geldreich et al. (1978) found that fecal coliforms lost viability faster than coliforms; however, the authors speculated that this was due to the diverse nature of the coliform group.
The premise for the Eijkman test is that incubation at the elevated temperature (44.5°C) will allow growth of organisms of fecal origin while inhibiting organisms of nonfecal origin. Although 92.7% of E. coli were positive by the Eijkman test, 7.8% of the nonfecal coliforms were also positive (Geldreich, 1966). Hendricks (1978) tested environmental isolates and noted that 25% of the Enterobacter and 35% of the Klebsiella strains tested by the Eijkman test were positive. Dutka (1979) found that incubation at 44.5°C also inhibited many of the fecal coliforms found in water. In a study involving 20 hospital isolates each of E. coli, Klebsiella spp., and Enterobacter spp., only 10–40% of the E. coli, 20–42% of the Klebsiella spp., and 10–20% of the Enterobacter populations were recovered when incubated at 44.5°C, depending on the medium used. Similarly, environmental isolates exhibited similar sensitivities to incubation at 44.5°C except that the inhibition was much more pronounced. In another study by Dutka (1979), organisms from a fresh fecal sample did not exhibit sensitivity to incubation at 44.5°C; however, after being resuspended in fresh lake water for 6 weeks at 20°C, the organisms exhibited a sensitivity characteristic of the environmental isolates (also see Xu et al., 1982). Thus, the fecal coliform group, although more specific than the coliform group, still consists of a heterogeneous population of bacteria that varies with the product examined as well as the temperature (44.5, 45.0, or 45.5°C) that is used for the Eijkman test (Weiss et al., 1983).
**Escherichia coli**

One of the major drawbacks of the coliform and fecal coliform groups as index organisms is their lack of fecal specificity. In part, the lack of specificity is due to the definition of coliforms which encompasses several genera of organisms and some members are not always associated with feces. Shortcomings of this definition were recognized as early as 1921 when Mackie (cited in Mack, 1977) proposed that oxidase-negative be incorporated into the definition of coliforms. Some 55 years later this same recommendation was made by Lupo et al. (1977). The addition of oxidase-negative to the definition of coliforms would eliminate the inclusion of lactose-positive *Aeromonas* sp. which are also oxidase positive. Additional characteristics, such as production of indole from L-tryptophan, inability to utilize citrate as a sole carbon source, a positive methyl red test, a negative Voges-Proskauer test, or inability to hydrolyze urea, have been suggested as additions to the definition of coliforms (Dufour, 1977). All of the above characteristics are possessed by most strains of *E. coli*, and it is without doubt the predominant coliform of the gastrointestinal tract. *E. coli* comprises about 95% of the coliforms present in feces (Dufour, 1977; Geldreich, 1978).

In addition to its predominance in feces, *E. coli* persists in the environment for periods equal to or greater than pathogens such as *Salmonella* (Geldreich, 1978). However, there is still much debate over the relative survival of *E. coli* in the environment, as compared to the survival of intestinal pathogens. In contrast to the report above, *E. coli* have been reported to lose viability more rapidly than pathogens
in food and water (Buttiaux and Mossel, 1961; McFeters et al., 1974). LeClerc et al. (1977) reported that *E. coli* die-off faster than salmonellae in water, but they suggested that an increase in sensitivity of *E. coli* detection methodologies may compensate for its poor survival outside the gut. The survival of *E. coli*, as compared to pathogens, in food and water needs to be studied further using modern detection methodologies (Xu et al., 1982).

In summary, none of the index organisms discussed are without flaws. Specific problems with each index group or organism have been discussed above. Additional drawbacks result from the definition of coliforms and the methods used to identify them. Inherent to the definition of fecal coliforms in water is the utilization of lactose with the production of acid or gas at 44.5°C. First, detection methods which are based upon the fermentation of lactose fail to detect strains of coliforms and *E. coli* which are incapable of fermenting lactose. Secondly, gas formation is dependent upon formic hydrogen lyase which has been shown to be sensitive to temperature (LeClerc et al., 1977) which may account for some of the 9 to 15% of *E. coli* negative for gas production when incubated at 44.5°C (Dufuor, 1977).

False positive coliform and fecal coliform reactions in water can be caused by nonfecal coliforms such as *Klebsiella* (Geldreich, 1978; Dutka, 1979). The proliferation of these nonfecal coliforms in water results in elevated counts but does not increase the health hazard. Therefore, water which does not represent a health hazard is often condemned. This can result in economic losses to individuals, communities, and industries such as those in the shellfish industry. Geldreich
(1978) suggested that direct monitoring for pathogens should be used for areas such as shellfish beds. Recent progress has made possible the recovery of many pathogens from the environment; although expensive, it may be cheaper to examine shellfish beds directly for specific pathogens (salmonellae and cytopathic viruses) than to experience losses incurred because of unwarranted condemnation of shellfish areas.

Despite the contrasting reports on the incidence and survival of \textit{E. coli} in the environment, it is probably our best index of the possible presence of intestinal pathogens in food and water. \textit{E. coli} has the greatest fecal specificity of the index organisms discussed, and it does not proliferate to any great extent outside the gut. Countries such as Denmark, Belgium, England, and France use \textit{E. coli} as their standard index organism. Acceptance of \textit{E. coli} worldwide has been hampered by the time and expense required to isolate and identify it specifically among other coliforms. New techniques which are rapid, sensitive, and inexpensive might resolve the drawbacks of \textit{E. coli} as the index organism of choice.

Detection Methods for Index Organisms

The standard methods for enumeration of coliforms, fecal coliforms, and \textit{E. coli} in water and wastewater are the multiple-tube fermentation technique and the membrane filtration test (APHA, 1985). When testing foods, the multiple-tube fermentation method or a direct-plating method using violet red bile agar may be used (APHA, 1984). All three methods have been modified over the years and are continually being improved.
In recent years, improvements in media and the addition of a resuscitation step have enabled greater recoveries of injured or stressed cells, and modifications of existing procedures have reduced the time and labor needed to obtain results. In addition, many rapid tests for the detection of coliforms and *E. coli* have been reported (Cundell et al., 1980; Goldschmidt and Fung, 1978; Hartman et al., 1986).

**Multiple-tube fermentation test**

The multiple-tube fermentation test consists of three sets of replicate tubes (usually 3 or 5 per set) which are each inoculated with three serial dilutions. Results are reported as the most probable number (MPN) which is based upon probability formulas. The number is an estimate of the mean density of coliforms in a sample. The MPN test is especially useful when testing commodities with low numbers of microorganisms such as food, water, and dairy products (APHA, 1984, 1985a, 1985b). The MPN test is imprecise; accuracy of the test can be increased by using 5 instead of 3 replicate tubes for each dilution (APHA, 1985).

MPN determinations are usually divided into three tests: the presumptive test, the confirmed test, and the completed test. In the presumptive test, samples are inoculated into a lactose-containing medium such as lauryl sulfate tryptone broth (LSTB). The presence of gas in the inverted vial at any time during incubation at 35°C for 48 h constitutes a positive test. Positive presumptive tubes are then confirmed by transfer to tubes of brilliant green lactose bile broth (BGLB) which are incubated at 35°C for 48 h. BGLB tubes showing gas production within
48 h are recorded as positive. When testing foods, the number of positive confirmed tubes is used to determine the confirmed MPN of coliform bacteria per gram or per milliliter of sample (APHA, 1984). When testing water, positive confirmed tubes are streaked on LES Endo agar plates and nutrient agar slants. Following incubation, typical colonies from the plates are inoculated back into LSTB to verify gas formation and a gram stain is made on cells from the nutrient agar slant. A positive completed test is indicated by gas formation in LSTB within 48 h and the presence of gram-negative, non-sporeforming, rod-shaped bacteria.

When testing for fecal coliforms and *E. coli* in water samples, positive presumptive tubes are transferred to tubes of EC medium and incubated at 44.5°C for 24 h. The production of gas indicates the presence of fecal coliforms. To identify *E. coli*, positive EC tubes are streaked on Levine's eosin methylene blue plates. Typical colonies are streaked on slants and confirmed by IMViC reactions, gram-stain, and formation of gas in LSTB.

The major disadvantage of the MPN test is the time, usually from 5 to 10 days, required to complete all the steps. The test is also labor-intensive requiring several transfers. Several investigators have reported that the 48-h incubation periods in the presumptive and confirmed tests can be reduced to 24 h each (Goepfert, 1976; Hastback, 1981). Hastback (1981) found that, of 89 shellfish samples, only 7 would have had a decrease in the reported MPN from greater than 230 to less than or equal to 230 fecal coliforms per 100 g of sample if the incubation of the presumptive medium was terminated at 24 rather than 48 h. Of
the resulting positive confirmed tests, 98% were obtained from presumptive media after 24 h of incubation. These results were confirmed by Dexter (1981) who noted no significant difference between the number of positive confirmed tests after 24 and 48 h presumptive tests. Samples of raw beef examined using a two-day MPN (24-h incubations for both the presumptive and confirmed tests) detected the same number of E. coli as the standard MPN procedure 98% of the time.

The detection and enumeration of coliforms by the MPN test is susceptible to interference by noncoliforms. The inhibitory nature of the medium is also thought to play a role in the failure to detect some coliforms (Evans et al., 1981b). Interference is thought to result from competition between noncoliforms and coliforms for available nutrients or from inhibitory products produced by the noncoliforms (Evans et al., 1981b). Water with standard plate counts exceeding 500 cells per ml have been shown to have reduced percentages of coliform positive samples (Geldreich, 1978). Similar trends were noted with hamburger (Goepfert, 1976). In a study of water and raw sewage, Evans et al. (1981b) reported that 80% of the samples containing coliforms exhibited interference; interference was noted in all three steps of the MPN test. By testing growth-positive, gas-negative presumptive tubes for the presence of coliforms and fecal coliforms, the MPN values for coliforms in chlorinated water samples (Evans et al., 1981b) and fecal coliforms in seawater (Olson, 1978) were significantly improved over numbers determined with the standard MPN procedure. Although this increased the accuracy of the test, these additional steps also increased the time, labor, and expense of an already slow and tedious procedure.
Because the MPN test is based upon the production of gas from lactose, anaerogenic strains of *E. coli* which make up about 5\% of the population (Edwards and Ewing, 1972) are not detected.

A rapid MPN test was developed by Andrews and Presnell (1972) for the examination of estuarine water. The test, which is now referred to as the "A-1 test," is a 24-h elevated-temperature test which is performed in A-1 broth. A-1 broth was formulated specifically for the growth of *E. coli*. A-1 broth consists of tryptone, lactose, NaCl, salicin, and Triton X-100. The A-1 test was later modified to include a 3-h resuscitation step at 35°C (A-1-M) (Andrews et al., 1979). The A-1 and A-1-M tests when used in the analysis of foods have compared favorably with existing methods (Andrews et al., 1975, 1979, 1981; Powell et al., 1979; Hunt et al., 1981). The A-1 test recovered numbers of *E. coli* from shellfish (Andrews et al., 1975) and frozen crab meat (Powell et al., 1979) equivalent to those recovered by APHA methods. In contrast, several investigators reported that the A-1 test was less effective than existing methods when shellfish (Yoovidhya and Fleet, 1981) and certain foods (Andrews et al., 1979, 1981) are examined. Andrews et al. (1979) enumerated fecal coliforms and *E. coli* in 11 different food types by using the A-1, A-1-M, and standard procedures. Means of fecal coliform and *E. coli* MPN values determined by the A-1 and A-1-M procedures were equal to or significantly greater than the means obtained by standard methods in a majority of the food types tested. The A-1-M test was more effective than the A-1 test in recovering fecal coliforms. Advantages of the A-1 test over standard MPN procedure include reductions in time,
materials, and labor. With further improvements, the A-1-M test may become the method of choice for examining food and water for coliforms, fecal coliforms, and E. coli.

More recently, Feng and Hartman (1982) described a rapid MPN procedure in which the fluorogenic substrate 4-methylumbelliferyl-β-D-glucuronide (MUG) was incorporated into LSTB. MUG does not fluoresce. When cleaved, however, 4-methylumbelliferone is released and exhibits a bluish fluorescence when irradiated with long-wave ultraviolet light. The enzyme β-glucuronidase cleaves the glucuronosyl-O-bond (Levvy and Marsh, 1960) of MUG releasing the fluorescent 4-methylumbelliferyl portion of MUG. Among the Enterobacteriaceae, only E. coli, some shigellae, salmonellae, and yersiniae hydrolyze MUG (Feng and Hartman, 1982; Petzel and Hartman, 1985). In one report, β-glucuronidase activity was detected in 97% of the E. coli tested (Kilian and Bulow, 1976). In the MUG-MPN procedure, E. coli was identified by the production of gas and fluorescence after 24 h of incubation at 35°C. Tests with water and foods exhibited few false-negative reactions. False-positive reactions ranged from 2 (Feng and Hartman, 1982) to 5% (Robison, 1984), depending upon the sample being tested. False-positive tests were caused by staphylococci (Moberg, 1985) and streptococci (Robison, 1984). The MUG-MPN procedure compared favorably with standard procedures in the analysis of foods. In frozen foods, a greater number of E. coli were detected by using MUG in LSTB than with standard procedures and in a shorter period of time (Moberg, 1985). Robison (1984) reported an agreement of 94.8% between standard methods and MUG in LSTB. An additional benefit of the
MUG-MPN is that anaerogenic E. coli are detected by this method since identification is not based solely on the production of gas from lactose. Additional benefits of this test are the simplicity, accuracy, low cost, and the short period of time required to complete testing.

A problem arises when the MUG test is used for foods, such as shellfish, that contain high levels of endogenous glucuronidase. Because large sample sizes are used in testing shellfish, the levels of glucuronidase in these products precludes use of MUG in presumptive media. MUG has been successfully incorporated into EC broth (Rippey and Chandler, 1985; Koburger and Miller, 1985), however, which saves 3 to 4 days in identification time when compared to standard procedures of shellfish examination. Koburger and Miller (1985) used an EC-MUG medium to confirm the presence of E. coli in oysters. No false-positives and only one false-negative was reported. Rippey and Chandler (1985) confirmed the presence of E. coli in 95.3% of fluorescent-positive tubes but found that 10.5% of the fluorescent-negative tubes contained E. coli. Thus, when testing shellfish, additional testing of fluorescent-negative tubes may be needed.

Membrane filtration technique

The second method approved for the detection of coliforms in water is the membrane filter technique (APHA, 1985b). The membrane filter test (MF) for coliforms is rapid and easy to perform. Results are reproducible and are more precise than the results obtained by the MPN procedure because numbers of colonies rather than positive tubes are
counted. The larger sample sizes used for the MF tests also increase the sensitivity and accuracy of the tests. MF tests have limited use, however, when testing turbid waters or waters containing large numbers of noncoliforms (APHA, 1985b). There are two recommended MF procedures: the direct procedure and the enrichment procedure. The direct procedure may be used when testing water which has consistently yielded results similar to those produced by the standard MPN methods. The enrichment procedure is used when examining water containing injured coliforms, such as chlorinated water. The enrichment procedure is the recommended method for the examination of potable water.

M-Endo broth or m-Endo agar are recommended for use in the MF test. They are buffered lactose media, also containing basic fuchsin dye. Lactose-fermenting bacteria produce acid which results in the formation of red colonies with a metallic green-sheen. The amount of water filtered depends upon the number of coliforms and noncoliforms suspected in the water sample. Generally from 100 to 500 ml of water are filtered through a 0.45-micron pore size membrane filter. The filter is then transferred to a plate containing either m-Endo agar or a sterile pad saturated with m-Endo broth. The plates are incubated at 35°C for 22 to 24 h. When an enrichment step is used, the filter is first placed on a pad saturated with LSTB and incubated for 1.5 to 2.0 h at 35°C. After incubation, coliforms are enumerated by counting dark-red colonies with a metallic green-gold sheen.

Fecal coliforms can also be enumerated by the MF technique. The procedures used are identical to those used for the enumeration of
coliforms with the exception of the culture medium used and the tempera-
ture of incubation. Instead of m-Endo, m-FC medium is used for the growth
of fecal coliforms. Similarly, m-FC medium can be used to saturate pads
when used as a broth or as a solid when agar is added to the medium.
The incubation temperature used for the enumeration of fecal coliforms
in water is 44.5°C; 45.5°C is used for foods. Since the temperature
of incubation is critical, plates are incubated in a plastic waterproof
bag which is submerged in a waterbath for 24 h. Following incubation,
fecal coliforms are enumerated by counting blue colonies. Usually, non-
fecal coliforms are not a problem when enumerating fecal coliforms because
of the selective nature of the incubation temperature and the bile salts
and rosolic acid present in m-FC. Because rosolic acid is inhibitory
to some fecal coliforms (Presswood and Strong, 1978), the reagent should
be omitted from the medium "if minimum background colony counts occur
and equivalent results are obtained without it" (APHA, 1985).

The MF test has often been criticized for its inefficiency for the
recovery of injured coliforms (Lin, 1973; Camper and McFeters, 1979;
McFeters et al., 1982). Improvements in the procedure and the media
used for enumeration have reduced this problem. McCarthy et al. (1961)
increased recoveries of injured coliforms by using an enrichment step
whereby filters were incubated on a nonselective medium to allow recovery
of injured organisms prior to exposure to the selective agents found
in m-Endo. Use of the enrichment step increased recoveries 100% over
the direct procedure. They also reported that m-Endo agar was less
inhibitory than pads saturated with m-Endo broth and that the concentra-
tion of fuchsin-sulfate in m-Endo could be reduced (m-Endo LES) without
loss of selectivity. Recoveries by the enrichment method using m-Endo LES have been shown to be comparable with results obtained by MPN techniques (Lin, 1973, 1976). Rose et al. (1975) used a two-layer enrichment method for the recovery of fecal coliforms from chlorinated river, reservoir, and marine waters. M-FC agar was used as the base-layer and lactose broth plus agar was used as the top-layer. After the filter was in place, the plates were incubated at 35°C for 2 h before transfer to a 44.5°C incubator for 22 to 24 h. Recoveries by the two-layer method averaged 93% higher than those by the direct m-FC procedure. Incubation for 5 h at 35°C prior to incubation at 44.5°C has been reported to be superior to the 2-h enrichment step (Green et al., 1980). Fecal coliform recoveries using the 5-h enrichment period were within the 95% confidence limits of the MPN estimate when chlorinated waters were examined.

Currently, m-Endo broth, and m-Endo and M-Endo LES agars are approved for use with the MF technique (APHA, 1985b). Despite improvements in the procedure and medium, recoveries of injured coliforms are low (Dutka, 1973; McFeters and Stuart, 1972; McFeters et al., 1982) and difficulty can be experienced in the distinction of coliforms from noncoliforms (Dutka, 1973; Evans et al., 1981a; Schiff et al., 1970). To eliminate these shortcomings, LeChevallier et al. (1983a) developed a membrane filtration medium, termed m-T7, which contains Tergitol 7 and polyoxyethylene ether W-1 as selective agents. Penicillin G was also added when additional selectivity was required. M-T7 recovered 86-99% more laboratory-injured coliforms, almost three times the number of coliforms from drinking water, and an average of 43% more coliforms from surface
and drinking waters than the standard MF procedure. When used for the enumeration of fecal coliforms, counts were 3.1 and 1.7 times greater than those obtained by standard m-FC and two-layer enrichment methods, respectively (LeChevallier et al., 1984). Plates were incubated for 8 h at 35°C before transferring them to 44.5°C; no background growth was noted. The maximum enrichment period for m-Endo was 4 h due to growth of noncoliforms. The advantages cited for m-T7 included: only one medium was required for both total and fecal coliform counts, no overlay agar was required, transfer of the filter was not necessary, and money was saved due to a minimum of manipulations.

Because several noncoliforms are capable of producing a metallic sheen on m-Endo (Evans et al., 1981a; Schiff et al., 1970), a procedure for the verification of coliforms was adopted (APHA, 1985b). The proportion of typical coliform colonies actually verified as coliforms ranged from 44-97% (Davenport et al., 1976; Dutka, 1973). Typical colonies are verified by gas production in BGLB broth, by inoculating a multi-test identification strip for Enterobacteriaceae, or by testing for β-galactosidase and cytochrome oxidase (APHA, 1985b). In the latter procedure, results are obtained in 4 h. The number of total coliforms verified was increased by 87% when tested for β-galactosidase and cytochrome oxidase rather than for gas production in LSTB, and of the β-galactosidase-positive and cytochrome oxidase-negative colonies identified, 90% were coliforms (LeChevallier et al., 1983b). The major advantage of the rapid test was that numbers of verified coliforms were determined within 28 to 30 h, whereas verification in BGLB lengthened the
MF technique to 3 days. There have been several reports on media superior to BGLB for the verification of coliforms (Evans et al., 1981a; LeChevallier et al., 1983b). Nonetheless, verification by lactose fermentation requires 24 to 48 h, eliminating the advantages of speed and simplicity of the MF technique. Thus, the accuracy, sensitivity, and speed of the MF technique are influenced by the efficiency of the verification procedure. New verification procedures which are more rapid and sensitive than those currently available are needed.

**Presence-absence test**

The presence-absence test has been proposed (Clark, 1968) as an alternative method for the detection of coliforms in drinking water (APHA, 1985b). The presence-absence test was more sensitive and economical than the MF test (Clark, 1968, 1969, 1980; Jacobs et al., 1986) and slightly more sensitive than the MPN procedure (Jacobs et al., 1986). Clark (1968) estimated that the cost of the presence-absence test was one-fifth that of the MF test. Unlike the MF and MPN tests, the presence-absence test was unaffected by high levels of noncoliforms (Clark, 1980); however, high levels of noncoliforms are infrequent in drinking water samples (Pipes et al., 1986). The presence-absence test also provides the opportunity for isolation of several indicators from a single medium whereas other methods required use of a different medium for each organism being investigated.

The presence-absence test could be used alone once established that results for a particular type of water sample were in agreement with
either the MF or MPN methods (APHA, 1985b). The procedure (APHA, 1985b) is simple to perform and requires few manipulations. Water samples (100 ml) are added to 50 ml of triple-strength presence-absence broth and incubated for 24 to 48 h at 35°C. Presence-absence broth consists of lactose broth, lauryl tryptose broth, with bromcresol purple added as a pH indicator. The production of a yellow color indicates that fermentation of lactose has occurred and coliforms are present. Positive cultures are verified by gas formation in BGLB broth within 48 h at 35°C. With the addition of rapid methods for verification of positive cultures, the presence-absence test could become the method of choice for the analysis of potable water.

**Direct-plating methods**

Like MF techniques, direct-plating methods for the recovery of coliforms provide quantitative results in a short period of time. Violet red bile agar (VRB) is recommended for coliform determinations in dairy products (APHA, 1985a) and other foods (APHA, 1984). In the standard procedure, a 1.0-ml sample or dilution thereof is transferred to a sterile plate and mixed with tempered VRB agar, and the agar is allowed to solidify. An additional layer of VRB agar is poured over the surface to inhibit surface colony formation. After the surface layer has solidified, the plates are inverted and incubated at 32°C for 24 h. Coliforms, which appear as dark red colonies, are counted following incubation. Because VRB agar is inhibitory to injured coliforms (Maxcy, 1973; Ray and Speck, 1973), a resuscitation step in a nonselective medium has been
included so that injured cells can repair before they are exposed to the selective agents (Hartman et al., 1975; Speck et al., 1975). The resuscitation step is generally carried out by mixing the sample in a base layer of standard methods agar (Hartman et al., 1975) or tryptic soy agar (Speck et al., 1975) and incubating the plate at room temperature for 2 h. The plates are then overlayed with VRB agar, the agar is then allowed to solidify, and the plates are incubated for 22 h at 32°C. Yields of coliforms were increased when the resuscitation procedure was used to test dairy products (Hartman, 1975; Ray and Speck, 1978; Reber and Marshall, 1982). When standard methods agar, rather than tryptic soy agar, was used as a base layer, counts often were higher because of the glucose present in standard methods agar (APHA, 1985a). Glucose has been reported to facilitate the repair of injured cells (Draughton and Nelson, 1981); however, higher counts may in part be due to the recovery of some nonlactose fermentors (APHA, 1985a). The recovery of injured *E. coli* without a resuscitation period was accomplished by adding 3,3'-thiodipropionic acid to VRB agar (McDonald et al., 1983). Other methods for the recovery of sublethally injured cells have been reviewed (Mossel and Corry, 1977; Hartman, 1979; Ray, 1979).

Nelson et al. (1984) developed a double-layered film (Petrifilm), which contains dry VRB in the bottom layer and a gelling agent with tetrazolium dye in the top layer, for coliform analysis. Coliforms are identified by the presence of gas bubbles adjacent to the colonies and a red coloration of the colonies due to the reduction of the tetrazolium dye. Numbers of coliforms recovered by the Petrifilm VRB were comparable to
those obtained by using VRB agar or the MPN technique (Nelson et al., 1984).

There have been several reports on the enumeration of fecal coliforms by incubation of VRB agar plates at 44.5°C (Klein and Fung, 1976; Powers and Latt, 1979; Stiles and Ng, 1980). In one series of experiments, recoveries of fecal coliforms were equivalent to those obtained by MPN and MF techniques when water samples were plated directly on VRB agar without resuscitation and incubated at 44.5°C (Klein and Fung, 1976). Stiles and Ng (1980), however, obtained higher fecal coliform counts from hamburger when a resuscitation step was included. VRB agar has also been used with a resuscitation step to recover freeze-injured *E. coli* (Powers and Latt, 1979). Alternatively, the fluorogenic-substrate MUG, which is specifically hydrolyzed by *E. coli* (Kilian and Bulow, 1976; Feng and Hartman, 1982) may be added to VRB agar to distinguish *E. coli* from other coliforms without incubation at elevated temperatures (Feng and Hartman, 1982; Alvarez, 1984; Moberg, 1984).

Anderson and Baird-Parker (1975) developed a rapid direct plating method for the enumeration of *E. coli*. A 1.0-ml sample was spread on a cellulose membrane resting on the surface of a plate of minerals modified glutamate agar. The plate was incubated for 4 h at 35°C to allow injured cells to repair (Holbrook and Anderson, 1982) and permit carbohydrates, which might otherwise inhibit indole formation (Beggs and Lichstein, 1965), to diffuse into the underlying agar. The membrane was then transferred to a plate of tryptone-bile agar and incubated for 18 h at 44°C. Following incubation, the membrane was removed, stained,
dried under UV light, and observed for the presence of indole positive colonies. In an analysis of 843 samples of poultry, meat, fish, and shellfish, 95% of indole-positive colonies were identified by IMViC reactions as *E. coli*, 3.4% were fecal coliforms, and 1.6% were noncoliforms. Advantages of the Anderson and Baird-Parker method include the short time required to obtain definite results, the ability to recover injured cells, numbers of *E. coli* are determined without further verification, and nonlactose fermenting and anaerogenic *E. coli* are recovered. Some of the disadvantages are that each batch of tryptone-bile agar must be tested to assure that klebsiellae are inhibited and sufficient indole is produced by *E. coli*, the membranes used are expensive, and indole reagent kills the cells which prevents further testing. If further testing is warranted, subcultures must be made. However, immunological confirmation tests which do not require living cells have not been tested.

Because of the inhibitory nature of VRB agar, Wright (1984) developed a lauryl sulphate-aniline blue (ELSAB) agar medium which is similar to the medium developed by Francis et al. (1974). Higher coliform counts were obtained with ELSAB agar than with VRB, MacConkey, or deoxycholate agars when fecal samples were examined. When food and water samples were tested, ELSAB agar recovered numbers of *E. coli* greater than or equal to those obtained with standard media. *E. coli* were identified by making subcultures which were tested for indole production.

Damare et al. (1985) developed a peptone tergitol glucuronide (PTG) agar for the enumeration of *E. coli*. The medium lacks carbohydrate and the inhibitory selective agents found in other selective media but
contains the specific indicator MUG for the identification of \textit{E. coli}. PTG agar was superior to VRB or tryptone bile agars for the recovery of heat- and freeze-stressed cells. \textit{E. coli} counts from poultry and meat were equivalent or greater than numbers determined by the MPN test. Of 224 fluorescent-positive isolates obtained on PTG agar, 222 were confirmed as \textit{E. coli}. Thus, Damare et al. (1985) placed the emphasis on the use specific indicators rather than selective agents as a means of enumerating \textit{E. coli}. Further work needs to be done on other indicators for the identification of index and indicator organisms.

**Rapid methods**

For many years it has been realized that methods more rapid than those currently used for total and fecal coliforms are needed. Ideally, the method should be inexpensive, simple, and sensitive. To date, a number of procedures have been described in the literature, but all are less than ideal.

The earliest methods for the rapid detection of coliforms utilized radioactive substrates (Levin et al., 1961; Waters, 1972; Bachrach and Bachrach, 1974). Coliforms were detected by capturing $^{14}\text{CO}_2$ released from the metabolism of labeled substrate and measuring the radioactivity in a liquid scintillation counter. The time needed to produce a detectable response for a particular sample was proportional to the logarithm of the initial cell count in the sample (Waters, 1972). Although rapid and sensitive, the radiometric tests require expensive equipment. Additional problems include the identification of stressed coliforms which have extended lag periods prior to division, the nonbiological
release of labeled CO₂ (Cundell, 1981), and a strong resistance in industry to adopt isotopic methodologies.

Two electrochemical methods for the detection of coliforms have been reported (Wilkins et al., 1974; Wilkins and Boykin, 1976; Silverman and Munoz, 1979). The first method is based upon the linear relationship between inoculum size and the time of hydrogen evolution (Wilkins et al., 1974; Wilkins and Boykin, 1976). Hydrogen production is detected by an increase in voltage between two electrodes. Wilkins and Boykin (1976) reported that a single cell of E. coli was detected in 7 h. An electrical impedance technique has been successfully used for the rapid detection of microorganisms in blood and urine (Cady et al., 1978). The basis of the test is that microorganisms break down nutrients, like carbohydrates, which are electrically inert, into large quantities of charged molecules and ions. The increase in conductivity due to the charged molecules causes a change in the electrical impedance. A linear relationship was found between the log₁₀ of the number of fecal coliforms in an inoculum and the time required for an electrical impedance signal to be detected. Silverman and Munoz (1979) noted that 10⁶ to 10⁷ fecal coliforms grown in a lactose-based selective broth medium at 44.5°C generated a detectable signal. An inoculum containing 100 fecal coliforms was detected in 6 to 8 h. For a more detailed discussion of the principles and applications of impedance methods, see Cady et al. (1978) and Firstenberg-Eden and Eden (1984).
Chromatographic techniques have been used for a number of years in clinical laboratories for the identification of bacteria. Bacteria are generally identified by observing the pattern of metabolic by-products like short-chain fatty acids. The resulting chromatographs obtained by these techniques are like "signatures" that can be used to identify specific bacteria (Moss, 1981). Newman and O'Brien (1975) used these techniques to identify total and fecal coliforms in water. By using gas chromatography, coliforms were identified by the production of ethanol in a lactose-based medium. Total coliform determinations were made at 37°C and fecal coliforms were identified by incubating at 44.5°C. Minimum detection times of 9 and 12 h were required for 5 coliforms per ml and 50 fecal coliforms per ml, respectively. Although chromatographic techniques have much promise, the initial cost of the equipment required and the complexity of the procedure itself are major drawbacks to the widespread use of this procedure.

The firefly luciferase ATP assay and the Limulus amebocyte lysate assay for endotoxin correlate the presence of total ATP and endotoxin present within a sample, respectively, to the numbers of bacteria present. The number of bacteria present is related to the general bacteriological quality of the product. The Limulus assay has been successfully used for quantifying gram-negative bacteria in stream water (Evans et al., 1978) and ground beef (Jay, 1977). Although these tests show promise in the rapid assessment of food and water quality, batches of Limulus lysate vary somewhat in the levels of endotoxin detected, and drawbacks to the ATP assay include endogenous ATP present in food and the high cost of the reagents and equipment required.
The use of enzyme profiles as a means of identifying microorganisms has been used indirectly for many years. Lactose fermentation, used to detect the presence of coliforms, depends upon the enzyme β-galactosidase. The use of enzyme assays for the rapid identification of bacteria have several advantages over standard procedures. Enzyme assays are more sensitive than standard procedures because the enzyme itself continually produces a detectable endproduct; thus, they can be more rapid because they may not require extensive growth of the organism.

As described earlier, typical coliform colonies on membrane filters may be verified by testing for β-galactosidase and cytochrome oxidase. β-galactosidase activity was detected by the hydrolysis of O-nitrophenyl-β-D-galactoside (ONPG). Similarly, a rapid fecal coliform test was described which provides an estimate of the fecal coliforms present within 8 to 20 h; a single cell requiring 20 h for detection (Warren et al., 1978). A water sample was filtered, incubated in EC medium for 1 h at 37°C, and transferred to 44.5°C at which time a sterile solution of ONPG solution was added. The time between the addition of ONPG and the time at which one-half the maximum absorbance was reached was proportional to the concentration of fecal coliforms present in the sample. A linear relationship between ONPG hydrolysis times and fecal coliform MPN values from stream and lakewater samples was established (Warren et al., 1978).

Glutamate decarboxylase has been used for the rapid identification of E. coli. The enzyme is found in E. coli, Proteus mirabilis, Shigella, Providencia, Bacteriodes, and some Clostridium species (Freier et al.,
1976). The enzyme cleaves glutamic acid to release CO₂ and α-aminobutyric acid. LeClerc et al. (1978) developed an automated procedure for the detection of *E. coli* in water. Water samples were filtered and concentrated during passage through a hollow fiber filter. The samples were incubated in a lactose broth at 41°C to enrich for *E. coli*. After the CO₂ was removed from the culture, glutamate in a low pH buffer was added. The CO₂ produced by glutamate decarboxylase was captured in a phenothalein solution where it was detected colorimetrically. A single *E. coli* cell in 100 ml of water could be detected by incubating the sample in the lactose broth for 10 h prior to assaying for glutamate decarboxylase activity.

The enzymes β-glucuronidase and tryptophanase which were alluded to earlier have been used for the identification of *E. coli*. The procedure of Anderson and Baird-Parker identifies colonies of *E. coli* by using an indole stain. Indole, pyruvate, and NH₃ are the byproducts of tryptophan degradation by the enzyme tryptophanase. Only 1% of *E. coli* fail to produce indole from tryptophan (Abiss and Blood, 1982). β-glucuronidase was reported to be present in 97% of the *E. coli* tested (Kilian and Bulow, 1976) and only a few salmonellae, shigellae, and yersiniae (Feng and Hartman, 1982; Petzel and Hartman, 1985). As described earlier, Feng and Hartman (1982) have incorporated the fluorogenic substrate MUG, which is specifically cleaved by β-glucuronidase, into standard coliform culture media for the rapid identification of *E. coli*.

Antibodies to specific microorganisms provide the base for some of the most rapid and sensitive identification techniques available.
Antibodies have been labeled with fluorescein isothiocyanate, isotopes, and enzymes in order to provide a means of quantifying or visualizing the antibody-antigen reaction.

The fluorescent antibody technique has been approved for *Salmonella* screening (APHA, 1984); however, positive samples must be confirmed by standard culture techniques because salmonellae antisera cross react with other members of the *Enterobacteriaceae* such as *Arizona* sp., *Citrobacter* sp., and *E. coli* (Thomason and Wells, 1971). Cross reactivity could not be removed without losing reactivity with *Salmonella* antigens, although when purified anti-salmonellae IgG was used one-half of the reactions with *E. coli* spp. and all the reactions with *Citrobacter* spp. were eliminated (Thomason and Wells, 1971). A membrane filter-fluorescent-antibody method was used to identify *E. coli* which were added to natural populations of bacteria in water (Guthrie and Reeder, 1969).

Abshire and Guthrie (1973) described a fluorescent antibody test for the detection of fecal pollution. Pooled antisera were prepared by mixing equal volumes of 10 antisera produced against 10 strains of *E. coli*. The pooled antisera cross-reacted with *Enterobacter aerogenes*, *Klebsiella pneumoniae*, *Arizona arizonae*, *Salmonella typhimurium*, *Salmonella schottmuelleri*, and *Staphylococcus aureus* when tested by the fluorescent antibody technique. However, after the antisera were adsorbed with *Enterobacter* and *Klebsiella*, there was a high correlation between EC-positive and fluorescence-positive cultures. In addition to problems with cross-reactivity, the fluorescent antibody test requires expensive equipment when automated, it is tedious to perform, and the results are often subjective.
Since Engvall and Perlmann (1971) described the use of an enzyme-linked antibody to quantify an antigen (immunoglobulin), the literature has been flooded with reports on rapid identification procedures for microorganisms, toxins, and immunoglobulins as well as methods for quantitating biologically important compounds (i.e., hormones). Although radioimmunoassays (antibodies labeled with isotopes) are rapid and very sensitive, their use is limited by the expense of the reagents and equipment needed, the short shelf life of the isotopes, and the hazards and disposal problems associated with the use of isotopes. A thorough review of the applications, procedures, and amplification of enzyme-linked immunosorbent assays (EIA) is beyond the scope of this review; however, several thorough reviews have been published (Voeller et al., 1976; Schuurs and Van Weemen, 1977; Maggio, 1980; Yolken, 1981; Tijssen, 1985; Swaminathan and Konger, 1986).

The potential use and benefits of EIA in the identification of food-borne bacteria is best demonstrated in current procedures for the identification of Salmonella in foods (Minnich et al., 1982; Mattingly and Gehle, 1984; Anderson and Hartman, 1985). These procedures are sensitive, specific, and much more rapid than the pure culture technique which requires 5 to 7 days to complete. The cross-reactivities noted with polyvalent OH antisera in the fluorescent antibody test were not detected when the IgG fraction of the same antisera was used in the EIA (Minnich et al., 1982; Anderson and Hartman, 1985). The salmonellae assay was further improved when monoclonal antibodies were substituted for the polyvalent antisera used (Robison et al., 1983; Mattingly and Gehle,
In addition, EIA has been used to detect several other food-borne pathogens and their toxins (Swaminathan and Konger, 1986).

A third method which has been used to detect antigen-antibody reactions is agglutination. Agglutination assays are gaining popularity in diagnostic microbiology because they provide a rapid, simple, and inexpensive method for detecting an antigen or organism of interest. Coagglutination is a sensitive agglutination method which is enhanced by attaching specific antibodies to an inert particle such as formalin-fixed *Staphylococcus aureus* cells, charcoal, erythrocytes, or latex particles. Binding to these particles aids in the visualization of the antigen-antibody complex. Although agglutination reactions are generally not considered to be as sensitive as EIA procedures, in one study latex agglutination was more sensitive than EIA and crossed-immunoelectrophoresis (McCarthy, 1985). Coagglutination kits are commercially available for the identification of beta-hemolytic streptococci, *Neisseria gonorrhoeae*, cryptococcal polysaccharide, pneumococci, *Haemophilus influenzae* (Tilton, 1981; Goldschmidt, 1981), *Staphylococcus aureus*, and salmonellae (Remel, Lenexa, KS). Additional coagglutination tests for shigellae (Goldschmidt, 1981), *Legionella pneumophila* (Tilton, 1981), rubella (Stevens, 1981), and several other viruses (Goldschmidt, 1981) have been described.

**Monoclonal Antibodies**

Antibodies have long been recognized as one of the most valuable diagnostic tools available to the clinical microbiologist. Because of
their specificity, antibodies have been used to identify, quantify, classify, and purify antigens or organisms of interest. However, conventional antisera often cross react with antigens of similar structure and vary in the quality and quantity of antibody obtained from animal to animal and often from one bleeding to the next from the same animal. As a result, conflicting results are often obtained between laboratories or between batches of antisera. The heterogeneous nature of antisera results from the stimulation of B-cells which each recognize a particular antigenic determinant on the injected antigen. Since most antigens have a number of determinants (a bacterium may have thousands), a population of B-cells are stimulated upon exposure to an antigen. Thus, immunization with most antigens results in a polyclonal response and the production of many antibodies with differing affinities, avidities, and specificities.

Early investigators were interested in obtaining a population of homogeneous antibodies in order to elucidate the structure of antibodies. Multiple myeloma, a malignancy of antibody-producing cells that occurs in mice and other mammals, provided an early source of homogeneous antibodies which were produced from a single clone or B-cell (monoclonal antibodies). Potter (1970) found that some of the myeloma proteins from tumor-bearing mice reacted with known bacterial antigens. Attempts to induce tumors in immunized mice that would secrete antibodies specific to the immunogen used for immunization failed. Virus transformation of antigen-stimulated B-cells, with viruses such as SV40 and Abelson virus, has been used to establish cell lines producing specific antibodies.
(Steinitz et al., 1977), but only small quantities of antibodies were produced.

Kohler and Milstein (1975) produced a stable cell line which secreted antigen-specific antibodies by fusing mouse myeloma cells with lymphocytes from the spleen of an immunized mouse. The antibodies produced by the resulting cell lines, called hybridomas, were homogeneous populations of a single antibody and represented one of the many antibody types elicited by the antigen. By using this technology, unlimited supplies of highly specific antibodies to a wide variety of immunogens have been produced.

Many modifications of the procedure originally described by Kohler and Milstein have been proposed, but the basic procedure is still the same. Spleen cells from immunized mice or rats are removed, mixed with myeloma cells, and fused by using polyethylene glycol. The mixture is then plated in a hypoxanthine-aminopterin-thymidine (HAT) medium which selects for hybrids resulting from the fusion of spleen and myeloma cells (Littlefield, 1964). The myeloma cell line used has been genetically altered so that the enzyme hypoxanthine-guanine phosphoribosyl transferase (HGPRT) is nonfunctional. HGPRT⁻ myeloma cells are unable to synthesize purines from hypoxanthine, and because aminopterin is present in the medium, the main synthetic pathway for purines and pyrimidines is blocked. Thus, the HGPRT⁻ myeloma cells survive only when fused to B-cells which complement the defective HGPRT. B-cells are not sensitive to aminopterin but are unable to grow in culture for more than a few days; immortality is acquired from the myeloma cell.
Once the hybrids have grown to a sufficient size, the culture medium is assayed for the presence of antibodies by using a rapid screening method such as EIA. Hybrids producing antibody with the desired characteristics are cloned to ensure that only a single population of antibody-secreting hybridomas is present and to avoid overgrowth by nonantibody producing variants. Subclones positive for antibody are expanded, frozen, and injected into mice for production of high-titer ascites fluid. More detailed descriptions of monoclonal antibody production and screening have been published (Fasekas de St. Groth and Scheidegger, 1980; Van Deusen and Whetstone, 1981).

Monoclonal antibodies have a number of advantages over polyclonal antisera. Firstly, once developed they provide an endless supply of well-defined antibody which greatly reduces variation between lots. Secondly, specific antibodies to immunogens which are difficult to purify can be generated; these antibodies can subsequently be used to purify the antigen by affinity chromatography. Thirdly, since only hybridomas which secrete antibodies with desired characteristics are maintained, there are generally fewer cross-reactions and no need for adsorption. Lastly, for continuous large-scale production of antibodies, hybridomas are less expensive than conventional methods. However, when only small amounts of antibody are required, such as for research purposes, conventional antisera may be preferable. Production of conventional antisera requires less effort and money when small amounts of antibody are needed. The production of a stable hybridoma is labor-intensive and requires 4 to 6 months when all goes well. Conventional sera can be obtained
in less than 2 months. Thus, when antibodies with restricted specificities are required, it may be easier to remove the cross-reacting antibodies from a polyclonal preparation by adsorption than to find a monoclonal antibody with the desired specificity.

Although in some situations the use of traditional antisera may be warranted, the advantages of monoclonal antibodies far exceed the initial labor and cost. For thorough discussions on the potential as well as successful applications of monoclonal antibodies in biology and medicine, a number of excellent reviews have been published (Kennett et al., 1984; Schonherr and Houwink, 1984; Yelton and Scharff, 1981).

In the remainder of this review, the use of monoclonal antibodies in the analysis of several antigens from a number of gram-negative bacteria will be discussed. Emphasis will be placed on the specificity of the antibodies produced, the distribution of the particular antigens among species and genera, and the uses of the antibodies produced.

Monoclonal antibodies to gram-negative bacteria

Within the outer membrane of gram-negative bacteria, lipopolysaccharides and proteins are the major antigenic constituents. Lipopolysaccharides (LPS) consist of lipid A, a core-region, and an 0 polysaccharide side chain (O-antigen). The O-antigens are diverse antigenically (Orskov et al., 1977) because the polysaccharide side chains vary in length and composition, or they are absent. The core and lipid A portions are somewhat more conserved both structurally (Jansson et al., 1981) and antigenically (Mutharia et al., 1985). Polyclonal antibodies
to lipid A cross reacted with purified lipid A and crude lipopolysaccharide from several Salmonella spp. and an E. coli sp. (Galanos et al., 1971). Monoclonal antibodies to lipid A from E. coli J5 and Pseudomonas aeruginosa reacted with membrane and LPS preparations from 36 strains of P. aeruginosa and 22 other gram-negative bacteria from the families Vibrionaceae, Enterobacteriaceae, Rhizobiaceae, and Pseudomonadaceae (Mutharia et al., 1985). In another study, most of the monoclonal antibodies produced against E. coli J5 endotoxin reacted with only E. coli J5, but some reacted with endotoxin from other E. coli and a few salmonellae. When tested with heat-killed bacteria, most of the antibodies reacted with Salmonella minnesota (which lacked O-antigens) and the parental E. coli strain to J5. Almost half of the monoclonal antibodies reacted with P. aeruginosa and a few bound to Klebsiella pneumoniae. In comparison, monoclonal antibodies to the core and O-antigens of P. aeruginosa reacted with only a few other P. aeruginosa strains (Mutharia et al., 1985); likewise, monoclonal antibodies to an outer core structure from E. coli recognized cores from several other E. coli strains (Peters et al., 1985). The results of these studies indicate that lipid A and the core portions of LPS contain one or more antigenic determinants conserved within a species. These determinants might not be accessible to antibodies unless the cells are heated or in some way treated to expose the core or lipid A structures.

Monoclonal antibodies have also been produced to the enterobacterial common antigen (ECA) which is found on the surface of all Enterobacteriaceae (Makela and Mayer, 1976). The antibodies detected
the ECA in heated-extracts from clinical isolates of E. coli, Enterobacter, Klebsiella, Proteus, Salmonella, Shigella, and Yersinia spp. and failed to react with Acinetobacter, Pseudomonas, Campylobacter, Vibrio, Haemophilus, Bordetella, or Bacteriodes strains; reactions were weak with whole or encapsulated bacteria (Peters et al., 1985). Further work needs to be done on the distribution of the ECA among other gram-negative bacteria.

Defining and identifying serotypes of bacteria requires highly specific antisera. Sometimes polyclonal sera must be extensively adsorbed in order to obtain the desired monospecificity. Identification of specific serotypes is important in epidemiological studies. For example, serotypes of Shigella responsible for outbreaks of diarrhea are difficult to type because numerous related antigens are shared between the genera of Shigella and Escherichia (Edwards and Ewing, 1972). Carlin and Lindberg (1985) used monoclonal antibodies specific to the O-antigens of Shigella flexneri and Shigella sonnei to identify specific serotypes. Monoclonal antibodies produced against heat-killed shigellae resulted in a wide range of specificities. Various antibody preparations recognized: species-specific determinants of S. flexneri and S. sonnei, serogroup determinants of S. flexneri, both strain and serogroup determinants simultaneously, undefined determinants common to most S. flexneri O-polysaccharides, and lipopolysaccharide core antigen from both rough and smooth strains of S. flexneri. The monoclonal antibodies were more specific than conventional adsorbed rabbit antisera using EIA. The IgG monoclonal antibodies were subsequently used in a coagglutination
test for typing clinical isolates of _S. flexneri_ and _S. sonnei_; IgM monoclonal antibodies were used directly in agglutination tests. All of the monoclonal antibodies proved to be specific for serotyping purposes.

Similarly, specific antibodies are required for the detection of the somatic antigen 0:1 of _Vibrio cholerae_ which causes cholera. The species all contain the same flagellar (H) antigen (Mukerjie, 1978) and the same R antigen which may be exposed naturally or by heat (Donovan and Furniss, 1982). Therefore, typing sera must be specific for the 0:1 determinant and free of antibodies to H and R antigens. Tests with commercial antisera produced variable results (Donovan and Furniss, 1982). The serogroup 0:1 contains a group-specific antigen A and two type-specific antigens B and C. Based on this, the serogroup can be divided into three groups: Ogawa, containing antigens A and B; Inaba, containing antigens A and C; and Hikojima, which contains all three (Holme and Gustafsson, 1985). Monoclonal antibodies have been produced which specifically recognize the group-specific antigen A and each of the type-specific antigens B and C; no cross-reactions were noted with a number of enteric bacteria and _Brucella abortus_ (Holme and Gustafsson, 1985). Polyclonal antisera cross-reacted with _B. abortus_ because this bacterium contains sugars in its O chains similar to those in _V. cholerae_ (Holme and Gustafsson, 1985). In addition, only with monoclonal antibodies was it possible to detect both the B and C antigens in Hikojima strains. Thus, monoclonal antibodies provided an effective means of serotyping _V. cholerae_ 0:1.
Monoclonal antibodies have also been used to serotype *Neisseria meningitidis* (Sugaswara, 1985). Monoclonal antibodies were produced to the three serogroups (Groups A, B, and C) associated with meningitis; eight serogroups exist in all. Several monoclonal antibodies had specificities for group A, B, and C lipopolysaccharide while others recognized specific serotypes within a serogroup. Cross-reactions with *E. coli* K92 and K1 were noted (Sugaswara, 1985) with monoclonal antibodies to groups C and B, respectively, due to their similar carbohydrate structures (Kasper et al., 1973). Typing was done by direct agglutination; laboratory strains as well as new isolates were readily agglutinated. A monoclonal antibody specific for the pili of group A meningococci was produced which reacted with all group A strains tested and did not cross-react with pili from *Neisseria gonorrhoeae*, *E. coli*, *Hemophilus influenzae* type b, *Salmonella typhi*, or *Streptococcus pneumoniae* (Sugaswara, 1985). When the anti-pili antibody was used as the labelled antibody and polyclonal antiserum as the capture, 21 of 25 samples from infected patients were positively identified; however, 25 of 25 were identified when the polyclonal antibodies were used as both the capture and labelled antibody (Sugaswara, 1985). It was speculated that the epitope recognized by the monoclonal antibody was not present on the four strains which were negative and that a cocktail of anti-pilus monoclonal antibodies might be more effective.

Studies on a variant pilus from *Neisseria gonorrhoeae* strain P9, using monoclonal antibodies, indicated that common determinants are found on all P9 pilus types and strain-specific determinants (Virji et al.,
Likewise, both common and strain-specific determinants have been identified on E. coli pili (Soderstrom, 1985). Since pili are important in attachment to host cells, their antigenic makeup is important in vaccine development and in the study of the infection process. For example, Sadowski et al. (1983) protected neonatal pigs and calves from death by toxic diarrhea by orally administering monoclonal antibodies to E. coli K99 pili. The antibodies did not react with any other E. coli components. Mice were also protected from E. coli 06:K13:H1 when injected with monoclonal antibodies to pili and the K13 capsular antigen (Soderstrom, 1985).

Monoclonal antibodies have been used to identify a common determinant on Salmonella flagella (Smith et al., 1979). The antibody (M467) recognizing the common determinant on the salmonellae flagellins is an IgA myeloma protein which was derived from a plasma cell tumor of a Balb/c mouse (Potter, 1970). The tumors were induced by injection of mineral oil and adjuvant. Subsequently, the M467 antibody was used in an EIA for the detection of salmonellae (Robison et al., 1983). The antibody reacted with a flagellar determinant found on 94% of the Salmonella organisms tested and did not cross-react with any of the enteric organisms tested (Robison et al., 1983). The assay successfully detected Salmonella organisms in contaminated food and in mixed cultures (Robison et al., 1983). The assay has been improved by combining M467 with monoclonal antibodies specific to the Salmonella strains which did not react with M467 resulting in a cocktail of antibodies reacting with all salmonellae (Mattingly and Gehle, 1984).
It appears that monoclonal antibodies to flagella and pili might be used in similar assays to identify index microorganisms. However, many natural isolates of *E. coli* and other index organisms fail to produce flagella and pili when cultured by standard techniques. Future work needs to focus on the distribution of flagellar and pilus determinants among different groups of bacteria and on cultural techniques and media which enhance flagella and pilus production.

Monoclonal antibodies have been produced to several outer-membrane proteins including the bacteriorhodopsin of *Halobacterium halobium* (Kimura et al., 1982), and lactose permease (Carrasco et al., 1982), Omp A (Gabay et al., 1985), Pho E (Van der Ley et al., 1985), and Lam B proteins (Gabay and Schwartz, 1982; Schenkman et al., 1983) from *E. coli*. Most of the studies focused on using monoclonal antibodies to determine the structure, function, and orientation of the protein within the bacterial membrane. With the exception of the Lam B protein, little has been done on the distribution of these proteins within other genera of bacteria. However, monoclonal antibodies to Omp A were reported to react with the Omp A proteins from several *E. coli* strains and *Salmonella* spp. Anti-Pho E antibodies reacted with Pho E proteins from various enterobacteria when genes for Pho E were transferred to *E. coli* K12 (Gabay et al., 1985).

The Lam B protein serves as a receptor to the bacteriophages lambda and K10 and is involved in the transport of maltose and maltodextran. It was detected in extracts from all wild type *E. coli* tested and some shigellae (Schwartz and LeMinor, 1975). Monoclonal antibodies recognized six different epitopes on the Lam B protein; four were located on the
distal or external portion and two on the inner portion of the protein (Schenkman et al., 1983). The antigenic determinants on the external portion of the protein varied among bacteria and even within E. coli. One antibody reacted with 10 of 11 E. coli tested and a few shigellae; others reacted with only a few of the E. coli strains (Bloch and Desaymard, 1985). The two monoclonal antibodies to the internal determinants reacted with all E. coli as well as several shigellae, Enterobacter, Citrobacter, Erwinia, and Klebsiella which contained the Lam B protein (Bloch and Desaymard, 1985). It appears that the monoclonal antibodies have the specificity needed to distinguish the Lam B of E. coli from the Lam B found in other enterobacteria; this distinction could not be made using polyclonal antibodies (Bloch and Desaymard, 1985). It was not determined if these antibodies could be used to detect Lam B in viable cells.

Antibody-based assays for microorganisms provide a rapid, sensitive, and inexpensive means of identifying microorganisms. Coupled with the specificity of monoclonal antibodies, it is feasible that a rapid test for the detection or confirmation of index organisms in food and water can be developed.
SECTION I.

COAGGLUTINATION AND ENZYME-CAPTURE TESTS FOR THE DETECTION OF

ESCHERICHIA COLI β-GALACTOSIDASE, β-GLUCURONIDASE, AND

GLUTAMATE DECARBOXYLASE
ABSTRACT

Polyclonal antibodies to *Escherichia coli* β-galactosidase, β-glucuronidase, and glutamate decarboxylase were used in coagglutination tests for identification of these three enzymes in cell lysates. Enzyme-capture assays were also developed for the detection of *E. coli* β-galactosidase and β-glucuronidase. The enzymes were released by using a gentle lysis procedure that did not interfere with antibody-enzyme interactions. All three enzymes were detected in 93% of the *E. coli* strains tested by coagglutination; two of the three enzymes were identified in the remaining 7%. Of 42 non-*E. coli* tested by coagglutination, only four nonspecifically agglutinated either two or three of the anti-enzyme conjugates. Thirty-two (76%) non-*E. coli* were negative by coagglutination for all three enzymes. The enzyme-capture assay detected the presence of β-galactosidase in seven of eight and β-glucuronidase in all eight strains of *E. coli* tested. Some strains of β-galactosidase-positive *Citrobacter freundii* and *Enterobacter cloacae* were also positive by the enzyme-capture assay, indicating that the antibodies were not entirely specific for *E. coli* β-galactosidase; however, five other gas-positive non-*E. coli* were negative by the enzyme-capture assay. The coagglutination tests and enzyme-capture assays were rapid and sensitive methods for the detection of *E. coli* β-galactosidase, β-glucuronidase, and glutamate decarboxylase.
INTRODUCTION

Because members of the coliform and fecal coliform groups have been found in the environment (APHA, 1985a, 1985b; Dutka, 1979; Geldreich, 1978), <i>Escherichia coli</i> is often used as an indicator of fecal contamination. <i>E. coli</i> is specific to the intestinal tracts of warm-blooded animals (APHA, 1985a; Dufour, 1977; Geldreich, 1978) and is usually present in numbers greater than other coliforms and pathogens (APHA, 1985a). However, the isolation of <i>E. coli</i> from a mixture of coliforms and verification of the isolates require considerable time and expense. Although improvements in <i>E. coli</i> detection have been made (Anderson and Baird-Parker, 1975; Feng and Hartman, 1982; LeClerc et al., 1977), additional methods that are more rapid, and methods which can be used to complement existing methods, are needed.

Enzyme immunoassay (EIA) and coagglutination tests have provided a rapid means of identifying bacteria (Goldschmidt, 1981; Mattingly and Gehle, 1984; Swaminathan and Konger, 1986; Tilton, 1981). Most of the EIA and coagglutination tests utilize antibodies directed to cell-surface somatic (O; Carlin and Lindberg, 1985), capsular (K), flagellar (H; Mattingly and Gehle, 1984; Minnich et al., 1982), or other surface antigens (Lam et al., 1985; Sugawara, 1985) which are common to the species being detected. Identification of a common surface determinant among all strains of <i>E. coli</i> is difficult because of the numerous serogroups (Orskov et al., 1977). <i>E. coli</i> also contain cross-reacting antigens, shared by other members of the family Enterobacteriaceae (Thomason, 1981).
Intracellular constituents of \textit{E. coli}, unlike the surface components (antigens), include several antigens that are common to \textit{E. coli}. Four antigens, $\beta$-galactosidase, $\beta$-glucuronidase, glutamate decarboxylase, and tryptophanase are produced by a majority of \textit{E. coli} (Edwards and Ewing, 1986; Freier et al., 1976; Kilian and Bulow, 1976). Previous studies with $\alpha$-amylase (Inone et al., 1965) and $\beta$-galactosidase (Nader et al., 1985) have demonstrated that similar enzymes from different sources are immunologically distinct (Inone et al., 1965; Nader et al., 1985). Therefore, this study was undertaken to determine if polyclonal antibodies could be used to detect \textit{E. coli} enzymes, and if these antibodies were capable of distinguishing between the enzymes produced by \textit{E. coli} and those produced by other prokaryotes and eukaryotes.
MATERIALS AND METHODS

Stock Cultures and Media

Environmental strains of *E. coli*, numbered 1 through 72 (Ochman and Salander, 1984), were obtained from S. A. Minnich (IGEN, Inc., Rockville, MD). Many of the non-*E. coli* cultures were obtained from P. Feng (IGEN, Inc., Rockville, MD). The remaining cultures were obtained from food and water samples and the culture collection at Iowa State University.

Media were commercial products (Difco laboratories, Detroit, MI), except for enzyme induction broth (EIB), which consisted of lauryl tryptose broth, 3.56 g; 4-methylumbelliferyl-β-D-glucuronide (MUG; Hach Co., Ames, IA), 0.015 g; L-glutamic acid, 0.05 g; distilled water, 100 ml; pH 6.8-7.0.

Antibody Production

*E. coli* enzymes used for immunization were obtained commercially (Sigma Chemical Co., St. Louis, MO). Rabbits were injected intramuscularly with 500 µg of β-galactosidase (G5635), β-glucuronidase (G1758), glutamate decarboxylase (G3757), or tryptophanase (T0754) in complete Freund's adjuvant. Four weeks after the initial injection, the rabbits received an identical dosage of enzyme in incomplete Freund's adjuvant. Finally, 500 µg of enzyme in 0.01 M of phosphate-buffered saline (PBS, pH 7.2) was administered intramuscularly 5 to 7 days prior to bleeding. Blood was collected by cardiac puncture. The sera were tested for anti-enzyme antibody by indirect enzyme immunoassay (Voeller et al., 1979).
Protein A Purification of IgG Antibodies

Blood was clotted overnight at 4°C, and the red blood cells were removed by centrifugation. Clarified serum was divided into aliquots and stored at -20°C. The IgG fraction was collected by using a protein A-Sepharose (Sigma) column. Before addition to the column, serum or ascites fluid was clarified by centrifugation for three minutes in a microfuge. Four milliliters of clarified serum or ascites fluid was added to 1.0 ml of 0.05 M PBS (pH 8.2), and the mixture was applied to an 8-cm x 1.25-cm protein A-Sepharose column equilibrated with 0.01 M PBS (pH 8.2). Eluate was monitored at 280 nm with an Isco model UA-2 ultraviolet analyzer. After unwanted serum proteins had passed through the column, as exhibited by a return to base line absorbance values, IgG was eluted with an acid solution (glacial acetic acid, 2.5 ml; NaCl, 4.5 g; NaN₃, 0.2 g; and distilled H₂O, 500 ml). When the pH of the eluate became acidic (shortly after an increase in $A_{280}$), 1-ml fractions were collected in tubes containing 0.2 ml of 0.5 M PBS (pH 8.2). Additional PBS was added if fractions were not completely neutralized. The fractions were collected until absorbance values returned to baseline values. The fractions were pooled, adjusted to pH 7.0, and stored in 2.0-ml volumes at -20°C. Once thawed, sodium azide was added to 0.2% and the preparations were stored at 4°C.

Quantities of IgG recovered from the protein A column were estimated by using $A_{280}$ values and an extinction coefficient of 1.4 (Kennedy and Dressman, 1983).
Enzyme Labelling of Antibodies and EIA

Alkaline phosphatase-antibody conjugates were prepared essentially as described by Voeller et al. (1976). Approximately 1.4 mg of IgG in 1.0 ml of 0.05 M PBS (pH 7.4) was mixed with 5 mg of type VII-T alkaline phosphatase (Sigma) and enough 25% glutaraldehyde to yield a final concentration of 0.2% (v/v). Following dialysis, 1% (w/v) bovine serum albumin (BSA) and 0.2% sodium azide were added to the conjugates. The conjugates were stored at 4°C.

Sandwich (Voeller et al., 1979) and indirect (Olsen and Rice, 1984; Voeller et al., 1979) EIAs were used to test for the presence of β-galactosidase, β-glucuronidase, and glutamate decarboxylase in crude enzyme preparations and cell lysates. In the indirect procedure, enzyme preparations and cell lysates were attached to microtitration plates (Nunc-Immuno plates; Hazelton Research Products, Denver, PA) by drying at 55°C.

Preparation of Staphylococcal Anti-enzyme Conjugates

To 300 μl of a 10% Staphylococcus aureus Cowan strain suspension (Sigma) in 0.05 M PBS (pH 7.5), 300 μg of one of the protein A-purified anti-enzyme antibodies was added. The volume of the mixture was adjusted to 1.0 ml with 0.01 M PBS (pH 7.2). The mixture was incubated at room temperature for 2 to 4 h with continuous agitation and then washed three times with 0.01 M PBS (pH 7.2). After the final wash, the staphylococcal-antibody conjugates were resuspended to 1.0 ml in 0.01 M PBS (pH 7.2) and sodium azide was added to 0.2%. The conjugates were stored at 4°C until used.
Cell Lysis

Four lysis procedures (Dobrogosz, 1981; Maniatis et al., 1982) were compared to determine the effects of various lysing agents on β-galactosidase activity. Cells from 24-h cultures grown in EIB were pelleted by centrifugation, resuspended to their original volume in 0.01 M Tris buffer (pH 8.0), and split equally into four tubes. The cells were again pelleted by centrifugation. The cell pellet in one tube was resuspended in Tris-HCl buffer containing 25 mM disodium ethylenedinitrilo tetraacetate (EDTA). Lysozyme and sodium dodecyl sulfate (SDS) were added to final concentrations of 5 μg/ml and 1%, respectively. The cell suspension was then incubated at room temperature for 10 minutes. The pellet in a second tube was resuspended in 0.01 M Tris-HCl buffer (pH 8.0) containing 25 M EDTA, 8% sucrose, and 0.5% Triton X-100 and was incubated in a 100°C waterbath for 45 seconds. Cells in the remaining two tubes were resuspended in distilled water. To one of the cell suspensions, one drop of toluene and four drops of 0.1% sodium deoxycholate were added. The tube was incubated at 37°C for 10 minutes and the pH neutralized with 35 μl of 1N NaOH. One hundred microliters of 3% KOH was added to the remaining cell suspension; after 5 minutes at room temperature, one drop of 1N HCl was added to neutralize the mixture. Following lysis, particulate material was removed by centrifugation, and 500 μl of 4-methylumbelliferyl-β-D-galactopyranoside (MUGAL, 100 μg/ml; Sigma) was added, and the mixture was incubated for 30 minutes at 37°C. Fluorescence under long-wave ultraviolet light was recorded as positive for the presence of β-galactosidase.
Based on the comparison of lysis procedures, an optimal method was developed. Cultures to be tested were grown for 24 h in EIB broth at 35°C. The cells from 0.5 ml were pelleted by centrifugation in a microfuge for 3 minutes. The cell pellet was resuspended to its original volume in lysis buffer (0.2 M Na₂HPO₄, 25 ml; 0.2 M NaHPO₄, 25 ml; sucrose, 8 g; EDTA, 0.93 g; Triton X-100, 50 μl; distilled H₂O, 50 ml; pH 7.0). Then an equivalent volume of RNAase solution (20 mg of Sigma bovine pancreas ribonuclease A per ml of 0.01 M PBS containing 0.05% Tween 20 and 3% BSA, pH 7.0) was added. A combination of ribonuclease and bovine pancreas deoxyribonuclease 1, both at 10 mg/ml, was also satisfactory. Finally, 15 μl of lysozyme solution (10 mg lysozyme/ml) was added to the cell pellet; the pellet was resuspended by agitation and incubated at 35°C for 20 minutes.

Coagglutination

To identify the enzymes by agglutination, 25 μl of cell-lysate was added to 25 μl of 0.01 M PBS (pH 7.0) on a glass slide, 12-15 μl of the respective staphylococcal-anti-enzyme conjugate was added, and lysates and conjugate were mixed. The mixture was allowed to react for 30-60 seconds and then rocked (1-2 minutes) until the E. coli control agglutinated. Agglutination reactions were observed with the aid of the lens of a bacterial colony counter.

Crude preparations of β-galactosidase, β-glucuronidase, and glutamate decarboxylase of various origins were obtained commercially (Sigma). The enzyme preparations were diluted to an equivalent of 0.5 enzyme units/ml of lysis buffer (pH 7.0). The diluted enzyme preparations
were mixed directly with 12-15 μl of staphylococcal-anti-enzyme conjugate and the agglutination procedure was performed as described above.

**Enzyme Capture**

Protein A-purified antibodies to *E. coli* enzymes were diluted in carbonate buffer (pH 9.6); 100 μl was added to a well of a microtitration plate (Nunc-Immuno plate I) and the plates were incubated at room temperature for 2 h or overnight at 4°C. The optimal dilution of antibody was determined by using 1:2 dilutions of the antibody and commercial enzyme diluted in lysis buffer. The highest dilution of antibody that produced a detectable reaction with a minimum quantity of enzyme (usually 1:500 to 1:1000 dilution) was used. Following attachment, the remainder of the antibody suspension was removed and remaining free binding sites were blocked with 250 μl of carbonate buffer containing 2% BSA for 1 h at room temperature. Plates coated with antibody could be stored at 4°C for up to one week.

After blocking the nonspecific binding sites, the plates were washed three times with 0.01 M PBS containing 0.05% Tween 20 (pH 7.4). Cultures to be tested were grown for 24 h in EIB broth and lysed. One hundred microliters of the cell lysate was added per antibody-coated well and the plates were incubated at room temperature with shaking for one hour. The plates were washed three times and then 100 μl of PBS, containing substrate for the enzyme captured, was added to each well. To detect the presence of captured β-galactosidase, either 4-methylumbelliferyl-β-D-galactopyranoside (MUGAL, 200 μg/ml) or O-nitrophenyl-β-D-
galactopyranoside 6-phosphate (ONPG, 300 µg/ml; Sigma) was added. β-glucuronidase was detected by adding either 4-methylumbelliferyl-β-D-glucuronide (MUG, 200 µg/ml) or p-nitrophenyl-β-D-glucuronide (PNPG, 300 µg/ml; Sigma). Plates were incubated with substrate for 30 minutes at 35°C. To stop reactions and enhance fluorescence, 100 µl of 0.2 N NaOH was added to each well. The A$_{409}$ of wells incubated with the colorimetric substrates, ONPG and PNPG, were determined by using a Dynatech Minireader. Wells incubated with MUG and MUGAL were read visually under long-wave ultraviolet light. Because reactions with MUG and MUGAL were more easily detected, subsequent tests were conducted only with the fluorogenic substrates.
RESULTS AND DISCUSSION

Detection of GAL, GUD, and GAD by EIA

A double-antibody sandwich EIA was developed to test cell lysates for GAL, GUD, and GAD. Dilutions of 1:500 for anti-GUD (1.34 mg/ml) and 1:1000 for anti-GAL (2.57 mg/ml) and anti-GAD (2.42 mg/ml) were optimum for use as capture antibody. Between 100-200 ng/ml of the respective commercial enzyme preparations could be detected (data not shown). Cultures tested by the sandwich EIA were grown overnight at 35°C in EIB and lysed with EDTA-lysozyme (Dobrogosz, 1981). Some enzyme was detected; however, the results varied and the background was high, probably because of nonspecific binding of the alkaline phosphatase anti-enzyme conjugates.

When an indirect EIA (Voeller et al., 1979) was used to test cell lysates, GAL and GAD were detected in all *E. coli* lysates (Table 1); *Pseudomonas fluorescens* which lacks GAL and GAD was positive in one experiment but not in the other. GUD was detected in only three of the four *E. coli* cultures tested, and absorbance values for the three positive cultures were low. Whether the variation between experiments in absorbance values was caused by differences in growth of the cultures or to the degree of lysis between experiments was not determined. Because these experiments were conducted with lysates that were not treated with nucleases, the lysates of *P. fluorescens* were viscous. This may account for the positive GAL and GAD reactions in the first experiment. The *E. coli* lysates were not viscous.
TABLE 1. Absorbances obtained when bacterial lysates were tested for β-galactosidase by using an indirect EIA

<table>
<thead>
<tr>
<th>Organism</th>
<th>Expt. 1</th>
<th></th>
<th></th>
<th>Expt. 2</th>
<th></th>
<th></th>
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<tbody>
<tr>
<td></td>
<td>GAL</td>
<td>GUD</td>
<td>GAD</td>
<td>GAL</td>
<td>GUD</td>
<td>GAD</td>
</tr>
<tr>
<td>E. coli ET3C</td>
<td>1.10</td>
<td>0.13</td>
<td>0.63</td>
<td>0.70</td>
<td>NT$^b$</td>
<td>0.11</td>
</tr>
<tr>
<td>E. coli KK2B</td>
<td>0.93</td>
<td>0.13</td>
<td>0.29</td>
<td>1.00</td>
<td>NT</td>
<td>0.26</td>
</tr>
<tr>
<td>E. coli 60</td>
<td>0.41</td>
<td>0.05</td>
<td>0.20</td>
<td>0.61</td>
<td>NT</td>
<td>0.08</td>
</tr>
<tr>
<td>E. coli B</td>
<td>1.35</td>
<td>0.13</td>
<td>0.60</td>
<td>1.14</td>
<td>NT</td>
<td>0.21</td>
</tr>
<tr>
<td>Pseudomonas flavescens</td>
<td>0.20</td>
<td>0.03</td>
<td>0.25</td>
<td>0.00</td>
<td>NT</td>
<td>0.00</td>
</tr>
</tbody>
</table>

$^a$The background has been subtracted from absorbance values.

$^b$Not tested.
Release of Intracellular Enzymes

Because GUD reactions were weakly positive and the results for GAL and GAD varied when tested by the indirect EIA, several additional lysis procedures were tested. To discover a lysis procedure that did not disrupt enzyme antigenic structure, four procedures were evaluated for their ability to release GAL in an enzymatic form. Cells were lysed by using EDTA-SDS-lysozyme, toluene-SDC, 3% KOH, and EDTA-Triton X-100-lysozyme. MUGAL was added to the lysates to detect enzyme activity. The EDTA-Triton X-100-lysozyme procedure released active GAL from all four *E. coli* and was most suitable for the purposes of this research. These results are in agreement with a previous report on the denaturing effects of detergents (Goding, 1983) on proteins. Non-ionic (Triton X-100), weakly ionic (SDC), and strongly ionic (SDS) detergents, in this order, correlate with increasing solubilizing power as well as disruption of protein-protein interactions and denaturation (Goding, 1983).

To further optimize the lysis procedure, tests were conducted to determine optimum concentrations of Triton X-100 and if the heating step was essential for lysis (data not shown). Incubation at 100°C for 45 seconds was both unnecessary and detrimental to the release of active GAL; therefore, incubation at 100°C was eliminated from the lysis procedure. Concentrations of 0.01%, 0.05%, and 0.10% of Triton X-100 released high levels of active GAL. A concentration of 0.05% Triton X-100 was used for subsequent lysis.
Detection of GAL and GUD by Enzyme-capture

Because colorimetric and fluorogenic substrates were available for GAL and GUD and both enzymes were active following lysis with EDTA-Triton X-100-lysozyme, an enzyme-capture assay was developed. The assay was easy to perform and could be completed within 90 minutes. Instead of adding a second anti-enzyme antibody, as in the sandwich EIA, or an alkaline phosphatase-conjugated anti-rabbit IgG, as in the indirect EIA, substrate to the captured enzyme was added. ONPG and MUGAL were used to detect the presence of captured GAL; PNPG and MUG were used to detect captured GUD. Reactions with MUGAL and MUG were stronger and more easily read than reactions with ONPG and PNPG. The sensitivity of the enzyme-capture method was excellent: 175 ng of purified GAL/ml produced a strong reaction when MUGAL was used as substrate.

Initially, GUD was not detected by the enzyme-capture assay. Although GUD activity was present in cell lysates, the enzyme was either not bound because it had been altered antigenically during lysis or it was not active when bound by antibody. Another contributing factor may have been that the substrate solution was not optimal for enzyme activity (Sakaguchi and Murata, 1984). Therefore, the lysis buffer was prepared in 0.1 M sodium phosphate buffer (pH 7.0) instead of Tris buffer, and the substrates MUG and PNPG were dissolved in 0.1 M PBS (pH 6.8) instead of distilled water. When these modifications were made, GUD was detected in cell lysates (data not shown).

Both GAL and GUD were detected in cell lysates when cells were lysed in phosphate-EDTA-Triton X-100-lysozyme (Table 2). Seven of the eight
TABLE 2. Production of gas from lactose, serological detection of GAL, cleavage of MUG, and serological detection of GUD

<table>
<thead>
<tr>
<th>Organism</th>
<th>GAS&lt;sup&gt;a&lt;/sup&gt;</th>
<th>anti-GAL&lt;sup&gt;b&lt;/sup&gt;</th>
<th>MUG&lt;sup&gt;c&lt;/sup&gt;</th>
<th>anti-GUD&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Citrobacter freundii</td>
<td></td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Citrobacter freundii</td>
<td>1404573</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Citrobacter freundii</td>
<td>TF146</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
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<td>Enterobacter aerogenes</td>
<td>MC12</td>
<td>-</td>
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<td>-</td>
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<tr>
<td>Enterobacter agglomerans</td>
<td></td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Enterobacter agglomerans</td>
<td>Carol 4</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Enterobacter cloacae</td>
<td>F31</td>
<td>+</td>
<td>±</td>
<td>-</td>
</tr>
<tr>
<td>Enterobacter cloacae</td>
<td>F32</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Escherichia coli</td>
<td>B</td>
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<td>+</td>
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<td>+</td>
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<td>Escherichia coli</td>
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<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Klebsiella oxytoca</td>
<td>Ohio 6</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Klebsiella ozanae</td>
<td>L901</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Klebsiella pneumoniae</td>
<td>WSSC 26</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Klebsiella pneumoniae</td>
<td>F29</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Klebsiella pneumoniae</td>
<td>Ohio 3</td>
<td>+</td>
<td>±</td>
<td>-</td>
</tr>
<tr>
<td>Shigella sp.</td>
<td>F21</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

<sup>a</sup>Gas production was recorded after incubation at 35°C for 24 h in EIB.

<sup>b</sup>Capture antibody to E. coli β-galactosidase (GAL); β-glucuronidase (GUD).

<sup>c</sup>The MUG reaction was determined after incubation at 35°C for 24 h in EIB. Fluorescence under long-wave ultraviolet light was recorded as positive.
E. coli tested were strongly positive for GAL; the eighth strain was weakly positive. Among the GAL-positive non-E. coli only Citrobacter freundii 1404573 and Enterobacter cloacae F32 yielded positive tests for GAL. Enterobacter cloacae F31 and Klebsiella pneumoniae Ohio 3 exhibited some GAL activity, but the reactions were weak. Citrobacter freundii TF146, Enterobacter agglomerans Carol 4, and Klebsiella pneumoniae F29 produced gas from lactose, but were negative by the GAL-capture assay. The polyclonal antibodies exhibited some specificity in binding, suggesting that antigenic differences exist between GALs from different bacterial genera. However, the quantity of GAL produced by the different cultures might also account for these differences. The hydrolysis of MUG and the presence of GUD were restricted to E. coli. Further tests on purified GAL and GUD from various bacterial genera as well as other origins are needed. In addition, we have produced monoclonal antibodies to E. coli GAL and GUD and have begun studies on the specificities of these antibodies.

Identification of GAL, GUD, and GAD by Coagglutination

Staphylococcal anti-enzyme conjugates were used to detect GAL, GUD, and GAD in E. coli lysates. The sensitivity of the coagglutination procedure was good when purified enzyme preparations were used although sensitivities varied between conjugates (data not shown). The GAL conjugate was the most sensitive, agglutinating as little as 50 ng of GAL/ml of lysis buffer. The GAD and tryptophanase (TRYP) conjugates were equally sensitive, agglutinating approximately 2-3 μg of GAD and TRYP/ml of lysis buffer, respectively. Agglutination reactions for GAL, GAD, and TRYP
were optimal at enzyme concentrations above 7.5 μg of enzyme/ml. The GUD conjugate was the least sensitive of the conjugates, requiring approximately 31 μg of GUD/ml for agglutination; concentrations of 500 μg/ml or higher were optimal. Because of the poor sensitivity, agglutination with the GUD conjugate was the most difficult of the four conjugates to visualize.

Concentrations of inducers required for induction of GUD, GAD, and TRYP were determined qualitatively by assaying cell lysates for the presence of each of the respective enzymes (data not shown). The highest levels of MUG (150 μg/ml) and L-tryptophan (1000 μg/ml) tested yielded the highest levels of enzyme after the shortest period of growth. GUD activity was detected in cell lysates within 4 h of inoculation; however, levels of GUD were much higher after 24 h of growth. TRYP activity was barely detectable after 7 h; levels were higher after 24 h of growth. Concentrations of 0.05%, 0.1%, and 0.50% glutamate all resulted in detectable GAD activity after 24 h of growth. Differences in the strength of the GAD reactions could not be determined. Because lactose was already present in EIB, an inducer for GAL was not added. GAL activity attained maximal levels 4 h after inoculation. Based on the results from enzyme induction tests, lauryl tryptose broth was supplemented with 0.05% glutamate, 0.1% L-tryptophan, and 150 μg of MUG/ml (EIB). Also, cultures were incubated for 24 h prior to testing.

TRYP was not detected by coagglutination in E. coli lysates despite addition of 0.1% L-tryptophan to EIB to induce the enzyme; however, the enzyme preparation used for immunization was readily agglutinated by
the staphylococcal-anti-TRYP conjugate (data not shown). Failure to
detect TRYP in _E. coli_ by coagglutination may have resulted from a low
titer of anti-TRYP antibodies because the TRYP preparation used for
immunization was not as pure as the other enzyme preparations.
Alternatively, TRYP production might have been repressed during growth
in EIB; Beggs and Lichstein (1965) reported that the production of TRYP
is repressed in the presence of carbohydrates. Because TRYP was not
detected in cell lysates, L-tryptophan was eliminated from EIB.

Coagglutination tests were used to examine 42 non-_E. coli_ belonging
to 25 different species (Table 3). Of 11 gas-positive bacteria, seven
were agglutinated by the GAL conjugate. The GAL conjugate also
agglutinated a strain each of _Citrobacter freundii_ and _Enterobacter aerogenes_ that were negative for gas production after 24 h; however,
the _E. aerogenes_ was positive for gas production after 48 h. The GUD
conjugate agglutinated cell lysates from _Acinetobacter calcoaceticus_,
_Citrobacter freundii_, _Enterobacter cloacae_, and _Serratia fonticola_, all
of which were MUG-negative. One unusual, MUG-positive strain of _Hafnia alvei_ was not agglutinated. The GAD conjugate agglutinated lysates of
one strain each of _E. cloacae_, _H. alvei_, and _S. fonticola_. GAD activity
in these species is probably uncommon because Freier et al. (1976) did
not detect GAD in the _Hafnia spp._, _E. cloacae_, and _Serratia spp._ that
they tested. _E. cloacae_ and _S. fonticola_ were the only non-_E. coli_ to
agglutinate all three conjugates. Thus, a small number of bacteria tested
produced a cross-reacting antigen or some other material that agglutinated
the staphylococcal-anti-enzyme conjugates. The apparent cross-reactivity
TABLE 3. Numbers of non-\textit{E. coli} examined and numbers producing positive reactions in five different tests

<table>
<thead>
<tr>
<th>Organism</th>
<th>Strains tested</th>
<th>GAS(^b)</th>
<th>anti-GAL(^a) conjugate</th>
<th>MUG(^c) conjugate</th>
<th>anti-GUD(^a) conjugate</th>
<th>anti-GAD(^a) conjugate</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acinetobacter calcoaceticus</td>
<td>2</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>Arizona sp.</td>
<td>1</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Bacillus cereus(^d)</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Bacillus thuringensis(^d)</td>
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<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Citrobacter freundii</td>
<td>2</td>
<td>1</td>
<td>2</td>
<td>0</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>Enterobacter aerogenes</td>
<td>1</td>
<td>0</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Enterobacter agglomerans</td>
<td>2</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Enterobacter cloacae</td>
<td>5</td>
<td>4</td>
<td>3</td>
<td>0</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Hafnia alvei</td>
<td>2</td>
<td>1</td>
<td>1</td>
<td>1(^e)</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>Klebsiella oxytoca</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Klebsiella ozanae</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Klebsiella pneumoniae</td>
<td>6</td>
<td>3</td>
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<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Proteus mirabilis</td>
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<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

*\(^a\)Coagglutination with Staphylococcal-anti-enzyme conjugate.*

*\(^b\)Gas production was recorded after incubation at 35°C for 24 h in EIB.*

*\(^c\)The MUG reaction was determined after incubation at 35°C for 24 h in EIB. Fluorescence under long-wave ultraviolet light was recorded as positive.*

*\(^d\)Sparse growth in EIB after 24 h at 35°C.*

*\(^e\)Very weak activity.
<table>
<thead>
<tr>
<th>Organism</th>
<th>Strains tested</th>
<th>GAS</th>
<th>anti-GAL conjugate</th>
<th>MUG</th>
<th>anti-GUD conjugate</th>
<th>anti-GAD conjugate</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pseudomonas aeruginosa</td>
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<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
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<td>Pseudomonas putida</td>
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<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
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<tr>
<td>Salmonella enteritidis</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Serratia fonticola</td>
<td>2</td>
<td>1</td>
<td>1</td>
<td>0</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Serratia liquefaciens</td>
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<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Serratia plymuthica</td>
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<td>0</td>
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<td>0</td>
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<td>0</td>
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<tr>
<td>Shigella flexneri</td>
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<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
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<tr>
<td>Shigella spp.</td>
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<td>0</td>
<td>0</td>
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<td>0</td>
</tr>
<tr>
<td>Staphylococcus aureus&lt;sup&gt;d&lt;/sup&gt;</td>
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<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Streptococcus fecalis&lt;sup&gt;d&lt;/sup&gt;</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Vibrio fluvalis</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Yersinia enterocolitica</td>
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<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
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<td>Total</td>
<td>42</td>
<td>11</td>
<td>1</td>
<td>9</td>
<td>4</td>
<td>3</td>
</tr>
</tbody>
</table>
may have been caused by a minor protein contaminant(s) in the enzyme preparations used for immunization.

Originally, lysates of pseudomonads and klebsiellae were viscous. This prevented proper mixing of the lysate with the anti-enzyme conjugate and resulted in a filamentous, agglutination-like reaction (data not shown). The addition of RNAase to the lysate reduced its viscosity and false-positive reactions were eliminated (Table 3).

Numbers of E. coli and non-E. coli lysates reacting with three, two, one, or none of the staphylococcal-anti-enzyme conjugates are shown in Table 4. Of the 55 E. coli lysates tested by coagglutination, 51 (93%) were agglutinated by all three conjugates and the remaining 7% were agglutinated by two of the three conjugates. Only two E. coli were negative for GAD, and one was negative for GAL and one was negative for GUD. Individually, the anti-enzyme conjugates agglutinated 96-98% of the E. coli tested, which agrees with reports on the distribution of GUD and GAD in E. coli (Freier et al., 1976; Kilian and Bulow, 1976).

Edwards and Ewing (1986) reported that 90% of E. coli were positive for GAL. Percentages of E. coli positive for GAL exceeded 90% in this study because 2 gas-negative E. coli were positive for GAL by coagglutination. Apparently, some gas-negative E. coli contain a non-functional GAL which is still recognized by the antibody. Similarly, lysates from five of six MUG-negative E. coli were positive when tested with the anti-GUD conjugate. Thus, coagglutination appears to detect the presence of enzyme(s) in some strains that are negative when examined by using conventional fermentation tests.
TABLE 4. Total numbers of *E. coli* and non-*E. coli* examined and numbers and percentages (in parentheses) producing a positive reaction with 3, 2, 1, or none of the staphylococcal-anti-enzyme conjugates

<table>
<thead>
<tr>
<th>Number of strains tested</th>
<th>Number of lysates agglutinated by the following number of conjugates</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>3/3</td>
</tr>
<tr>
<td><em>E. coli</em></td>
<td>55</td>
</tr>
<tr>
<td>Non-<em>E. coli</em></td>
<td>42</td>
</tr>
</tbody>
</table>

*a* Cultures identified as *Serratia fonticola* and *Enterobacter cloacae* agglutinated all 3 anti-enzyme conjugates.

*b* *Citrobacter freundii* and *Hafnia alvei* agglutinated the anti-GAL and -GUD conjugates and the anti-GAL and -GAD conjugates, respectively.

*c* *Citrobacter freundii*, *Klebsiella pneumoniae*, *Enterobacter aerogenes* and 2 strains of *Enterobacter cloacae* agglutinated the anti-GAL conjugate; one strain of *Acinetobacter calcoaceticus* agglutinated the anti-GUD conjugate.
Among the 42 non-\textit{E. coli} (Table 4), only \textit{S. fonticola} and \textit{E. cloacae} (5%) agglutinated all three conjugates; \textit{C. freundii} and \textit{H. alvei} (5%) agglutinated two of the three anti-enzyme conjugates. Six (14%) of the non-\textit{E. coli} were agglutinated by a single conjugate, five by the GAL conjugate and one by the GUD conjugate (Table 4). This was not unexpected because 11 of the non-\textit{E. coli} tested were GAL positive (produced gas from lactose). The results show that the coagglutination test can specifically detect GAL, GUD, and GAD in \textit{E. coli} lysates.

The specificities of the coagglutination tests were examined further by using a variety of commercial enzymes (Table 5). The enzyme preparations were impure and contained other cellular constituents; therefore, preparations were diluted to an equivalent of 5 enzyme units/ml. The anti-GAL conjugate did not agglutinate GAL from \textit{Saccharomyces fragilis} or bovine liver but did agglutinate GAL from \textit{Aspergillus niger} and \textit{E. coli}. The anti-GUD conjugate agglutinated only \textit{E. coli} GUD. The anti-GAD conjugate agglutinated GAD from \textit{Clostridium welchii} and \textit{E. coli} equally well. These results and those described above indicate that polyclonal antibodies differ in their ability to distinguish between enzymes from different origins.

The significance of distinguishing between the \textit{E. coli} GUD and GUD from other sources is that some foods, such as shellfish, contain endogenous GUD. When large samples of these foods are added to media containing MUG, endogenous enzymes cause fluorescence in all tubes. Therefore, the examination of shellfish for \textit{E. coli} is restricted to the use of MUG in confirmatory EC media (Koburger and Miller, 1985). By using an
<table>
<thead>
<tr>
<th>Source and enzyme</th>
<th>Agglutination with staphylococcal-anti-enzyme conjugate</th>
<th>anti-GAL</th>
<th>anti-GUD</th>
<th>anti-GAD</th>
</tr>
</thead>
<tbody>
<tr>
<td>E. coli GAL</td>
<td>+</td>
<td>NA(^a)</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>Saccharomyces fragilis GAL</td>
<td>-</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>Bovine liver GAL</td>
<td>-</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>Aspergillus niger GAL</td>
<td>+</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>E. coli GUD</td>
<td>NA</td>
<td>+</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>Bovine liver GUD</td>
<td>NA</td>
<td>-</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>Chlamys opercularis GUD</td>
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<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>Abalone entralis GUD</td>
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<td>-</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>E. coli GAD</td>
<td>NA</td>
<td>NA</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>Clostridium welchii GAD</td>
<td>NA</td>
<td>NA</td>
<td>+</td>
<td></td>
</tr>
</tbody>
</table>

\(^a\)Not applicable.
antibody specific for *E. coli* GUD, tests might be made directly from presumptive tubes to obtain a one-day assay.

The coagglutination test also has promise as a rapid confirmatory test for colonies from agar plates or membrane filters. Preliminary results showed that colonies could be resuspended in a small volume of lysis buffer and RNAase solution on a glass slide and then tested with the anti-enzyme conjugates. Thus, colonies could be verified within minutes by testing for GAL, GUD, and GAD. Verification may require the use of only one conjugate, possibly GUD or GAD, because GAL-positive colonies are usually identified directly on lactose-containing differential media. The coagglutination and enzyme-capture procedures could also be used to verify the presence of *E. coli* in MPN tubes. Because of the simplicity and rapidity of the assays, all growth positive MPN tubes could be examined to increase the accuracy of the procedure (Evans et al., 1981; Olson, 1978).

Both the coagglutination test and the enzyme-capture assay provide rapid and sensitive means for the detection of GAL, GUD, and GAD. Tests of mixed cultures and food and water samples are needed, however, to determine the practical effectiveness of these procedures for the identification of *E. coli*. 
LITERATURE CITED


SECTION II.

PRODUCTION AND SPECIFICITY OF MONOCLONAL AND POLYCLONAL ANTIBODIES TO ESCHERICHIA COLI
Monoclonal and polyclonal antibodies were produced to heat-treated
*Escherichia coli*. The monoclonal antibodies were produced from Balb/c
mice immunized with a pool of five strains of heat-treated *E. coli*
The resulting hybridomas were tested by using *E. coli* strains other than
those used for immunization to facilitate detection of hybridomas pro-
ducing antibody that reacted with a large number of *E. coli* strains.
From 864 hybridomas, 32 reacted strongly with either two or all three
of the strains used for screening; 15 were successfully cloned. Antibody
from hybridoma 6H2 reacted with 35 of 68 (51%) *E. coli*; of 13 non-*E. coli*
tested, only *Enterobacter agglomerans* was positive. Monoclonal antibodies
from a hybridoma 9B12 reacted with all 6 *E. coli* tested and weakly with
*Enterobacter cloacae*; however, 9B12 stopped producing *E. coli*-specific
antibody. Five hybridomas produced antibodies that reacted with a major-
ity of the bacteria tested. Polyclonal antibodies produced against two
strains of *E. coli* differed in the numbers of *E. coli* and non-*E. coli*
detected when tested by enzyme immunoassay. Attempts to remove cross-
reacting antibodies were unsuccessful.
INTRODUCTION

Coliforms, fecal coliforms, and *Escherichia coli* are all used as index organisms for monitoring food and water (APHA, 1984, 1985a, 1985b). Although there is much debate over which index organism(s) provides the best signal for the presence of a health hazard (Hoadley and Dutka, 1977; Mossel, 1978, 1982), only *E. coli* is specific to the gastrointestinal tract of warm-blooded animals (Dufour, 1977; Geldreich, 1978). This specificity sets *E. coli* apart from the coliforms and fecal coliforms as an indicator of fecal pollution (Caplenas and Kanarek, 1984; Dutka, 1979; Geldreich, 1978; Weiss et al., 1983). Shortcomings of *E. coli* as an index organism are the time and expense required to isolate it from mixed culture and identify it.

Current methodologies for the detection of *E. coli* are laborious and time-consuming, often taking 96 h to complete (APHA, 1984, 1985a). Immunological tests, such as enzyme immunoassay (EIA) and coagglutination, provide a rapid means of detecting microorganisms from clinical and environmental samples (Goldschmidt, 1981; Mattingly and Gehle, 1984; Swaminathan and Konger, 1986; Tilton, 1981). Because of the large number of *E. coli* serotypes (Orskov et al., 1977) and the presence of cross-reacting antigens in *Enterobacteriaceae* (Thomason, 1981), it is difficult to obtain *E. coli*-specific antibodies. A fluorescent antibody technique has been described for the detection of *E. coli* in water (Abshire and Guthrie, 1973; Guthrie and Reeder, 1969). Although cross-reactions with *Enterobacter aerogenes, Klebsiella pneumoniae, Arizona*
Arizonae, Salmonella typhimurium, Salmonella shottmuelleri, and Staphylococcus aureus were reported, adsorption with cross-reacting bacteria reduced or removed reactions with the corresponding bacteria; reactions with E. coli were not affected (Abshire and Guthrie, 1973). The problems with the fluorescent antibody test are that it is tedious to perform, difficult to automate, and the results are often subjective.

The present study was undertaken to determine if monoclonal or polyclonal antibodies to E. coli could be used in an enzyme immunoassay (EIA) to detect E. coli. Monoclonal and polyclonal antibodies of varying specificities were produced.
MATERIALS AND METHODS

Stock Cultures and Media

Isolates of \textit{E. coli} numbered 1 to 72 (Ochman and Salander, 1984) were obtained from S. A. Minnich (IGEN, Inc., Rockville, MD). Many of the non-\textit{E. coli} cultures were obtained from P. Feng (IGEN, Inc., Rockville, MD). The remaining bacterial cultures were obtained from food and water samples and the culture collection at Iowa State University.

All media were commercial products from Difco Laboratories, Detroit, MI.

Preparation of Heat-treated Cells

Bacteria were grown overnight at 37°C in Brain Heart Infusion broth. One-tenth of a milliliter from the overnight culture was spread over the surface of each of eight Trypticase Soy Agar plates. The plates were incubated overnight at 37°C. Cells were harvested in sterile saline, pelleted by centrifugation, and resuspended in 20 ml of sterile saline. A portion was removed to determine cell numbers and the remainder incubated for 1 h at 100°C. The heated-cells were tested for sterility, diluted to approximately \(10^9\) cells per milliliter, and stored at 4°C.

Alternatively, bacteria were incubated overnight in Brain Heart Infusion broth at 37°C, pelleted by centrifugation, resuspended to their original volume in saline, and incubated for 1 h at 100°C. Unused portions of these heat-treated cell preparations were discarded.
Polyclonal Antisera

Rabbits were immunized weekly with increasing intravenous doses (0.5 to 2.0 ml) of sterile heat-treated *E. coli* (approximately $10^8$ cells per dose). After 4 weeks, blood was collected by cardiac puncture and the serum tested for *E. coli* antibodies.

Hybridoma Production

Balb/c mice were injected twice intraperitoneally with a pool of heat-treated cells from five strains of *E. coli* (culture numbers 60, 61, 62, 63, and 64). Injections were made 60 days apart; $10^8$ total cells were administered at each injection. Similar bacterins were administered intravenously 21 and 35 days after the last intraperitoneal injection. Four days after the last injection, spleens were harvested for fusion.

Spleen cells from immunized mice were fused with mouse plasmacytoma cell line Sp2/0, using methods previously described (De St. Groth and Scheidegger, 1980; Van Deusen and Whetstone, 1981). Spleen cells from two mice were washed and divided into equal portions containing between $5 \times 10^7$ to $3 \times 10^8$ cells. Each portion was fused with $5 \times 10^7$ washed Sp2/0 cells in polyethylene glycol 1500 (Boehringer and Mannheim Biochem., Indianapolis, IN). Cells were diluted to about $5 \times 10^5$ cells per milliliter in Iscoves modified Dulbecco's medium containing 20% macrophage conditioned medium and then were plated at $5 \times 10^4$ cells per well. The following day, an equal volume of the same medium containing hypoxanthine-aminopterin-thymidine was added to each well. After 9 to 12 days, hybridoma supernatants were assayed for the presence of antibody by EIA (Olsen and Rice, 1984).
Hybridomas producing desired antibodies were cloned by limiting-dilution directly from 96-well tissue culture plates. Clonings were made in Iscoves modified Dulbecco's medium supplemented with hypoxanthine and thymidine. After the first cloning, hypoxanthine and thymidine were omitted from the growth medium. Cloning was repeated three times or until all wells containing hybrids were positive for antibody production.

Protein A Purification of IgG Antibodies

Blood was clotted overnight at 4°C, red blood cells were removed by centrifugation, and the clarified serum was divided into aliquots and stored at -20°C. To isolate the IgG fraction, serum or ascites fluid was clarified by centrifugation for 3 minutes in a microfuge, 4.0 ml was added to 1.0 ml of 0.5 M phosphate buffered saline (PBS; pH 8.2) and the mixture was passed through an 8-cm x 1.25-cm protein A-Sepharose (Sigma Chem. Co., St. Louis, MO) column equilibrated with 0.1 M PBS (pH 8.2). Eluate was monitored at 280 nm with an Isco UA-2 ultraviolet analyzer. After elution of unwanted serum proteins, as exhibited by a return to baseline absorbance values, IgG was eluted with an acid solution (2.5 ml of glacial acetic acid, 4.5 g of NaCl, 0.2 g of NaN₃ in 500 ml of distilled water). When the pH of the eluate became acidic (shortly after an increase in A₂₈₀), 1-ml fractions were collected in tubes containing 0.2 ml of 0.5 M PBS (pH 8.2). Additional PBS was added if fractions were not completely neutralized. The fractions were collected until absorbance values returned to baseline values. Fractions were pooled, adjusted to pH 7.0, and stored in 2.0-ml aliquots at -20°C.
When an aliquot was thawed, sodium azide was added to 0.2% and the antibody preparation was stored at 4°C.

EIA

An EIA similar to the procedure described by Olsen and Rice (1984) was used to screen the hybridomas and determine the binding properties of the antibodies. The assays were made in 96-well, flat-bottomed Nunc-Immuno plates (Hazelton Dutchland, Inc., Denver, PA). First, 100 µl of heat-treated cell preparation was added per well and the plates were incubated at 55°C until dry (6 to 7 h was adequate, however, some plates were incubated overnight). These plates could be stored at room temperature for at least 3 days. For use, the wells were blocked with 250 µl of 2% bovine serum albumin (BSA) in 0.05 M carbonate buffer (pH 9.6) for 1 to 2 h at room temperature and then washed three times with 0.01 M PBS containing 0.05% Tween 20, pH 7.4. Purified antibody preparations were diluted in antibody diluent (0.01 M PBS containing 1% BSA and 0.05% Tween 20; pH 7.4). One hundred microliters of hybridoma supernatant or diluted antibody was added per well, and the plates were incubated at room temperature for 2 to 4 h and 1 h, respectively. The plates were washed three times, and then 100 µl of an alkaline phosphatase-conjugated anti-mouse or anti-rabbit immunoglobulin (Sigma) diluted in antibody diluent was added. When testing hybridoma supernatants, the plates were incubated with the conjugate at room temperature for 4 h or overnight. When purified antibodies or ascites fluid was used, the plates were incubated with the conjugate for only 1 h. Plates containing conjugate
were washed five times in wash buffer and then 100 μl of substrate buffer (diethanolamine, 10 ml; MgCl₂ 6H₂O, 10 mg; thimerosal, 25 mg; distilled H₂O, 90 ml; pH 9.8) containing 1 mg of p-nitrophenyl phosphate (Sigma) per ml was added to each well. After incubation at room temperature for 30 to 45 minutes, the absorbance of each well at 409 nm was determined with a Dynatech Minireader.

An alternative EIA procedure was developed in which the heating and drying steps were performed simultaneously. Cells from an overnight broth culture were pelleted by centrifugation and resuspended in 0.01 M PBS (pH 7.4). Tests were also made directly on broth cultures. One-hundred microliters of cell suspension or broth culture was added per well and the plates were heated in a microwave oven until dry (maximum output; approximately 30 minutes). A beaker of water was included so the cell suspension did not heat too quickly and boil out of the wells. The remaining steps of the EIA were carried out as described previously.

The optimal concentrations of purified-antibodies to be used in EIA were determined by using a series of 1:2 dilutions of antibody in antibody diluent and the E. coli strain used for immunization. The highest antibody dilution that produced a strong response visually by EIA and produced little background was used in subsequent EIAs.

Adsorption of Antiserum

Approximately 10⁸ heat-treated Citrobacter freundii, K. pneumoniae F29, and Enterobacter cloacae F31 (in 0.2 to 1.0 ml) were added to 17 μl of the E. coli 33 antiserum which was brought to a final volume of 6.7 ml
(1:400) in antibody diluent. The antiserum was incubated with the heat-treated cells for 15 minutes at room temperature. The antiserum, with the heat-treated cells still present, was then tested by EIA. Unadsorbed serum was diluted (1:400) in antibody diluent.

In addition, antiserum to E. coli 33 with C. freundii, C. freundii TF146, C. freundii 1404573, E. aerogenes, E. cloacae Igen 7, and K. pneumoniae F29 was tested by EIA as described above.
RESULTS AND DISCUSSION

Specificities of Monoclonal Antibodies to E. coli

Hybridomas were produced from Balb/c mice immunized with a mixture of heat-treated E. coli. Fusions with spleens from two mice immunized with these heat-treated E. coli resulted in 864 hybrids. When the hybridomas were screened by EIA with the mixture of E. coli used for immunization, variable results were obtained probably because an insufficient quantity of each strain was bound to the microtiteration plates. Therefore, the hybridomas were screened by using a single strain of E. coli that had not been used for immunization. This procedure facilitated the detection of hybridomas that produced antibody that reacted with strains of E. coli that had not been used for immunization.

Screening of the 864 identified 468 positive hybridomas (Table 1). Of these, 286 reacted with E. coli 33. Further testing with two natural isolates of heat-treated E. coli (TM1A and EK3C) resulted in 338 and 170 positive hybridomas, respectively. The number of positives identified with EK3C may have been low because smaller quantities of antigen were used. Some of the antibodies reacted with only one or two of the test strains, whereas others reacted with all three.

Of the 84 hybridomas that reacted with all three test strains, 32 produced an A_{409} greater than 1.0 for two or three strains. These 32 hybridomas were cloned by limiting-dilution; 15 (46%) were cloned successfully. Of the 15 cloned hybridomas, 10 produced IgG and four produced IgM (data not shown).
Table 1. Numbers of hybridomas that secreted antibodies that reacted with *E. coli* strains 33, TMIA and EK3C, as detected by EIA

<table>
<thead>
<tr>
<th>E. coli strain(s)</th>
<th>Number of hybridomas secreting reactive antibody</th>
</tr>
</thead>
<tbody>
<tr>
<td>33 only</td>
<td>75</td>
</tr>
<tr>
<td>TMIA only</td>
<td>116</td>
</tr>
<tr>
<td>EK3C only</td>
<td>35</td>
</tr>
<tr>
<td>33 + TMIA</td>
<td>107</td>
</tr>
<tr>
<td>33 + EK3C</td>
<td>20</td>
</tr>
<tr>
<td>TMIA + EK3C</td>
<td>31</td>
</tr>
<tr>
<td>33 + TMIA + EK3C</td>
<td>84</td>
</tr>
<tr>
<td>Total</td>
<td>468</td>
</tr>
</tbody>
</table>
Binding properties of nine IgG and one IgM (6H2) monoclonal antibodies were tested by EIA (Table 2). Data for three IgM monoclonal antibodies were not included in Table 2 because high levels of nonspecific binding were encountered; BSA and fetal bovine serum were used unsuccessfully to reduce nonspecific binding. It is interesting that three IgM antibodies were involved in nonspecific binding yet a fourth was not. Data from one IgG monoclonal antibody are not shown in Table 2 because the reactions were weak. Supernatants from cloned hybridomas and heat-treated cells of six _E. coli_ strains and 13 non- _E. coli_ were used. Negative controls included PBS, supernatant from the myeloma line Sp2/0 (negative for antibody production), and supernatant from hybridoma MAG 1 which produces antibody specific to maize dwarf mosaic virus.

The antibodies exhibited a wide variety of specificities (Table 2). Antibodies from hybridoma B215 reacted only with _E. coli_ EK3C. Antibodies from hybridomas 2H3, 6H9, 7E4, 8E5, and 9A10 produced strong reactions with a majority of the bacteria tested. Evidently, these bacteria contain a common determinant or cross-reacting antigen(s) which is recognized by the monoclonal antibodies. This phenomenon is common among gram-negative bacteria (Jenson et al., 1985; Mutharia et al., 1984). Nonspecific capture or attachment of the antibodies seems unlikely. Neither wells coated with PBS nor heat-treated cells were positive when reacted with either hybridoma supernatants or monoclonal antibodies to maize dwarf mosaic virus. Therefore, it seems that drying heat-treated cells to microtitration plates either exposed a widely distributed antigenic determinant recognized by the monoclonal antibodies or bound a
Table 2. Absorbancies obtained with 10 monoclonal antibodies when pure cultures of heat-treated bacteria were analyzed by EIA

<table>
<thead>
<tr>
<th>Organism</th>
<th>Absorbance at 409 nm</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>B215</td>
</tr>
<tr>
<td>Arizona sp.</td>
<td>0.00</td>
</tr>
<tr>
<td>Citrobacter freundii</td>
<td>0.00</td>
</tr>
<tr>
<td>Enterobacter agglomerans</td>
<td>0.00</td>
</tr>
<tr>
<td>Enterobacter cloacae</td>
<td>0.00</td>
</tr>
<tr>
<td>Escherichia coli B</td>
<td>0.06</td>
</tr>
<tr>
<td>Escherichia coli EK3C</td>
<td>0.19</td>
</tr>
<tr>
<td>Escherichia coli TM1A</td>
<td>0.00</td>
</tr>
<tr>
<td>Escherichia coli 31</td>
<td>0.04</td>
</tr>
<tr>
<td>Escherichia coli 33</td>
<td>0.06</td>
</tr>
<tr>
<td>Escherichia coli 34</td>
<td>0.00</td>
</tr>
<tr>
<td>Klebsiella pneumoniae</td>
<td>0.00</td>
</tr>
<tr>
<td>Proteus mirabilis</td>
<td>0.00</td>
</tr>
<tr>
<td>Pseudomonas aeruginosa</td>
<td>0.00</td>
</tr>
<tr>
<td>Salmonella enteritidis</td>
<td>0.00</td>
</tr>
<tr>
<td>Serratia liquefaciens</td>
<td>0.00</td>
</tr>
<tr>
<td>Serratia odorifera</td>
<td>0.00</td>
</tr>
<tr>
<td>Shigella flexneri</td>
<td>0.00</td>
</tr>
<tr>
<td>Shigella sonnei</td>
<td>0.00</td>
</tr>
<tr>
<td>Yersinia enterocolitica</td>
<td>0.00</td>
</tr>
<tr>
<td>Controls^</td>
<td>0.00</td>
</tr>
</tbody>
</table>

^See text.
cross-reactive material which binds or captures the monoclonal antibodies. Further testing of these monoclonal antibodies with various gram-negative bacteria is warranted.

The binding of antibodies from hybridomas 4F1, 6H2, 7A9, and 9B12 was restricted. Antibodies from hybridomas 6H2 and 9B12 were most specific for *E. coli*. Unfortunately, hybridoma 9B12 stopped producing *E. coli*-specific antibody. Ascites fluid produced after injection of 9B12 into Pristane-primed mice also lacked anti-*E. coli* antibody. Hybridoma 6H2 antibodies reacted with four of the six *E. coli*, *Enterobacter agglomerans*, and weakly with a *Shigella* sp. Further testing with 6H2 antibodies resulted in positive reactions for 35 of 68 (51%) *E. coli* strains tested (data not shown). Thus, production of monoclonal antibodies specific to *E. coli* is possible. However, additional monoclonal antibodies would be required to identify most strains of *E. coli*. The production of monoclonal antibodies to strains that failed to react with 6H2 antibodies should result in the production of hybridomas useful in the detection of the nonreacting strains. Such hybridomas should be screened with two strains of *E. coli* other than those used for immunization as well as a non-*E. coli* (to eliminate hybridomas that produce cross-reaction antibodies).

**Polyclonal Antibodies to *E. coli***

Polyclonal antibodies were produced against *E. coli* strains 33 and B. Antiserum to *E. coli* 33 was used in an EIA to determine which of five methods of heat and drying was most effective (Table 3). Unheated
Table 3. Absorbancies obtained by EIA using antiserum to *Escherichia coli* 33 and five different methods of heating and drying

<table>
<thead>
<tr>
<th>Organism</th>
<th>Not heated, dried</th>
<th>Heated in broth dried</th>
<th>Heated in PBS dried</th>
<th>Microwaved in broth</th>
<th>Microwaved in PBS</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Citrobacter freundii</em></td>
<td>0.30</td>
<td>0.44</td>
<td>0.28</td>
<td>0.41</td>
<td>0.39</td>
</tr>
<tr>
<td><em>Enterobacter agglomerans</em></td>
<td>0.07</td>
<td>0.21</td>
<td>0.53</td>
<td>0.16</td>
<td>0.40</td>
</tr>
<tr>
<td><em>Enterobacter cloacae</em></td>
<td>0.00</td>
<td>0.41</td>
<td>0.31</td>
<td>0.36</td>
<td>0.56</td>
</tr>
<tr>
<td><em>Escherichia coli B</em></td>
<td>0.06</td>
<td>0.29</td>
<td>0.91</td>
<td>0.57</td>
<td>0.77</td>
</tr>
<tr>
<td><em>Escherichia coli EK3C</em></td>
<td>0.00</td>
<td>0.31</td>
<td>0.29</td>
<td>0.28</td>
<td>0.30</td>
</tr>
<tr>
<td><em>Escherichia coli PK1B</em></td>
<td>0.02</td>
<td>0.41</td>
<td>0.34</td>
<td>0.39</td>
<td>0.50</td>
</tr>
<tr>
<td><em>Escherichia coli 33</em></td>
<td>0.42</td>
<td>0.78</td>
<td>1.16</td>
<td>1.07</td>
<td>0.47</td>
</tr>
<tr>
<td><em>Escherichia coli 56</em></td>
<td>0.02</td>
<td>0.40</td>
<td>1.02</td>
<td>0.34</td>
<td>0.67</td>
</tr>
<tr>
<td><em>Klebsiella ozanae</em></td>
<td>0.02</td>
<td>0.31</td>
<td>0.38</td>
<td>0.27</td>
<td>0.64</td>
</tr>
<tr>
<td><em>Klebsiella pneumoniae</em></td>
<td>0.04</td>
<td>0.39</td>
<td>0.28</td>
<td>0.35</td>
<td>0.47</td>
</tr>
<tr>
<td><em>Salmonella enteritidis</em></td>
<td>0.04</td>
<td>0.26</td>
<td>0.31</td>
<td>0.50</td>
<td>0.62</td>
</tr>
<tr>
<td><em>Serratia liquifaciens</em></td>
<td>0.03</td>
<td>0.28</td>
<td>0.11</td>
<td>0.35</td>
<td>0.50</td>
</tr>
</tbody>
</table>

^a^ Background values, as measured in control wells, were subtracted from absorbance values.

^b^ Cultures were heated at 100°C for 1 h in the growth medium prior to drying overnight at 55°C.

^c^ Cells were pelleted by centrifugation, resuspended in PBS, heated for 1 h at 100°C, and dried overnight at 55°C.

^d^ Cultures were added directly to wells of a microtitration plate and dried in a microwave oven.

^e^ Cells were pelleted by centrifugation, resuspended in PBS, added to wells of a microtitration plate, and dried in a microwave oven.
cells, with the exception of *C. freundii* and *E. coli* 33, failed to produce a positive reaction. None of the heating procedures was superior. Compared with heating in broth, three of the five *E. coli* tested produced a stronger reaction when heated in PBS. The other cultures varied similarly. When a microwave oven was used to simultaneously heat and dry the bacteria in broth, 10 of 11 bacteria reacted less strongly than cultures suspended in PBS. However, the reactions of cultures suspended in PBS and heated and dried in a microwave oven generally were equivalent to those heated in tubes and then dried at 55°C. Since results obtained by using the microwave procedure compared favorably with results obtained by the other procedures (Table 4), and the microwave procedure was rapid, subsequent EIAs using polyclonal antisera were performed by resuspending the test cultures in PBS and heating them in a microwave oven until dry.

To determine if polyclonal antibodies were as suitable as monoclonal antibodies for immunological detection of *E. coli*, several experiments were conducted with polyclonal antibodies. The specificities of two polyclonal antisera to *E. coli* were tested by EIA (Table 4). Antiserum to *E. coli* 33 reacted strongly with all of the bacteria tested except *Serratia liquifaciens*. The cross-reactivities with antiserum against *E. coli* 33 (07:*K*-:*H11*) may have been caused by the lack of *K* antigens in *E. coli* 33 which permitted access to cross-reacting antigens. In contrast, antiserum to *E. coli* B (rough:*K99*:nonmotile) was positive only for *E. agglomerans*, *E. coli* B, *E. coli* 56, and *Klebsiella ozanae*. The specificity of this polyclonal antiserum may be attributed in part to the presence of *K99* because *E. coli* 56 (06:*K99*:H1) was the only *E. coli*
Table 4. Absorbancies obtained with two polyclonal antisera when cultures of heat-treated bacteria were analyzed by EIA

<table>
<thead>
<tr>
<th>Organism</th>
<th>Absorbance at 409 nm(^a)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>anti-</td>
</tr>
<tr>
<td></td>
<td>E. coli 33</td>
</tr>
<tr>
<td><strong>Citrobacter freundii</strong></td>
<td>0.23</td>
</tr>
<tr>
<td><strong>Enterobacter agglomerans</strong></td>
<td>0.52</td>
</tr>
<tr>
<td><strong>Enterobacter cloacae</strong></td>
<td>0.29</td>
</tr>
<tr>
<td><strong>Escherichia coli B</strong></td>
<td>0.70</td>
</tr>
<tr>
<td><strong>Escherichia coli PK3C</strong></td>
<td>0.27</td>
</tr>
<tr>
<td><strong>Escherichia coli PK1B</strong></td>
<td>0.33</td>
</tr>
<tr>
<td><strong>Escherichia coli 33</strong></td>
<td>1.07</td>
</tr>
<tr>
<td><strong>Escherichia coli 56</strong></td>
<td>0.97</td>
</tr>
<tr>
<td><strong>Klebsiella ozanae</strong></td>
<td>0.33</td>
</tr>
<tr>
<td><strong>Klebsiella pneumoniae</strong></td>
<td>0.26</td>
</tr>
<tr>
<td><strong>Salmonella enteritidis</strong></td>
<td>0.28</td>
</tr>
<tr>
<td><strong>Serratia liquefaciens</strong></td>
<td>0.09</td>
</tr>
</tbody>
</table>

\(^a\)Background values, as measured in control wells, were subtracted from absorbance values.
positive by EIA (Table 4). *E. coli* EX3C (O not identifiable:K88:H7), *E. coli* PK1B (not typed), and *E. coli* 33 (O7:K-:H11) were negative when tested with antiserum to *E. coli* B. Thus, the selection of immunization strain(s) used for production of *E. coli* antiserum should be made carefully.

Since the antiserum to *E. coli* 33 reacted with all of the *E. coli* tested (Table 4), attempts were made to remove the cross-reacting antibodies by adsorption (Table 5). Adsorption reduced the intensity of reactions with *Citrobacter*, *Klebsiella*, *Enterobacter*, *Serratia*, and *Shigella*, but it also reduced the intensity of reactions with *E. coli*. Because the reactions with the non-*E. coli* tested were still too strong, three strains of *Citrobacter freundii* (Table 5), two *Enterobacter* spp. (Table 5; strain Igen 7, other not shown), and *Klebsiella pneumoniae* F29 were added to anti-*E. coli* 33 antiserum. Adsorption with all six non-*E. coli* together further reduced the reactions with *C. freundii* 1404573, *E. cloacae* WSSC 31, and *E. coli* PM1B. Reactions with other *Citrobacter* and *Enterobacter* remained high. Although adsorption of cross-reacting antibodies from *E. coli* antiserum has been reported (Abshire and Guthrie, 1973), reactions caused by cross-reacting antibodies in the present study could not be reduced to a satisfactory level without concomitant reductions in reactivity with *E. coli*.

The results of these studies suggest that polyclonal antisera are not suitable for use in an EIA procedure to identify *E. coli*. Results with monoclonal antibodies, however, suggest that these antibodies can be highly specific for *E. coli*. With additional monoclonal antibodies
Table 5. Absorbancies obtained by EIA using *Escherichia coli* 33 antiserum with and without addition of cross-reacting bacteria

<table>
<thead>
<tr>
<th>Organism</th>
<th>Absorbance at 409 nm&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Expt. 1</th>
<th>Expt. 2</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>anti-EC33&lt;sup&gt;b&lt;/sup&gt;</td>
<td>anti-EC33A&lt;sup&gt;c&lt;/sup&gt;</td>
<td>anti-EC33A&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td>Citrobacter freundii</td>
<td>0.46</td>
<td>0.20</td>
<td>0.16</td>
</tr>
<tr>
<td>Citrobacter freundii TF146</td>
<td>0.55</td>
<td>0.20</td>
<td>0.19</td>
</tr>
<tr>
<td>Citrobacter freundii 1404573</td>
<td>0.65</td>
<td>0.26</td>
<td>0.19</td>
</tr>
<tr>
<td><strong>Enterobacter agglomerans</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Carol 4</td>
<td>0.31</td>
<td>0.10</td>
<td>0.08</td>
</tr>
<tr>
<td>SL160</td>
<td>0.40</td>
<td>0.11</td>
<td>NT&lt;sup&gt;e&lt;/sup&gt;</td>
</tr>
<tr>
<td>F31</td>
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<td>0.10</td>
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<td>Igen 7</td>
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<tr>
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<tr>
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<td>35</td>
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<tr>
<td><strong>Shigella sp.</strong> F21</td>
<td>0.10</td>
<td>0.04</td>
<td>0.01</td>
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</table>

<sup>a</sup> Background values, as measured in control wells, were subtracted from absorbance values.

<sup>b</sup> Antiserum to *Escherichia coli* 33.

<sup>c</sup> Antiserum to *Escherichia coli* 33 to which 0.2 ml of heat-treated *Citrobacter freundii*, *Klebsiella pneumoniae* F29, and *Enterobacter cloacae* F31 were added.

<sup>d</sup> Antiserum to *Escherichia coli* 33 to which *Citrobacter freundii* (0.5 ml), *Citrobacter freundii* TF146 (1.0 ml), *Citrobacter freundii* 1404573 (1.0 ml), *Enterobacter aerogenes* (not shown, 0.3 ml), *Enterobacter cloacae* Igen 7 (1.0 ml), and *Klebsiella pneumoniae* F29 (0.3 ml) were added.

<sup>e</sup> Not tested.
to *E. coli*, immunological tests for the identification of most strains of *E. coli* could become a reality.
LITERATURE CITED


SUMMARY AND DISCUSSION

In Section 1, polyclonal antibodies to _E. coli_ GAL, GUD, and GAD were used in a coagglutination test for detection of each of these enzymes in cell lysates. The enzymes were released by using a gentle lysis procedure that did not alter antibody-enzyme interactions. All three enzymes were detected in 93% of the _E. coli_ strains tested by coagglutination; two of three enzymes were identified in the remaining 7%. Of 42 non- _E. coli_ tested, only four nonspecifically agglutinated either two or all three of the anti-enzyme conjugates. The remaining non- _E. coli_ (76%) were negative by coagglutination for all three enzymes. An enzyme-capture assay was also developed for detecting GAL and GUD in cell lysates. GAL was detected in seven of eight _E. coli_ and GUD in all eight _E. coli_ tested. Some strains of GAL-positive _Citrobacter freundii_ and _Enterobacter cloacae_ were also positive by enzyme-capture and coagglutination tests, indicating that the antibodies were not entirely specific for _E. coli_ GAL. Future studies on water and food samples are needed to determine the effectiveness of these procedures for identification of _E. coli_. Furthermore, monoclonal antibodies might be used in place of the polyclonal antibodies to further increase the specificity of the tests.

In Section II, the specificities of monoclonal and polyclonal antibodies to heat-treated _E. coli_ were determined by EIA. The monoclonal antibodies varied in their specificity. However, one stable hybridoma (6H2) was identified that reacted with 51% of the _E. coli_ tested and
weakly with a single strain of *Enterobacter agglomerans*. Five hybridomas produced antibodies that reacted with a majority of the bacteria tested. Polyclonal antibodies produced to two strains of *E. coli* also differed in the numbers of *E. coli* and non-*E. coli* detected by EIA. Attempts to remove cross-reacting antibodies by adsorption were unsuccessful. Future studies should focus on the development of additional monoclonal antibodies to *E. coli* strains that failed to react with antibodies from hybridoma 6H2.
APPENDIX

Production of Monoclonal Antibodies

The procedure used for the production of monoclonal antibodies was a combination of the methods described by Fasekas de St. Groth and Scheidegger (1980) and Van Deusen and Whetstone (1981) with some modifications. Below are the media, reagents, and procedures necessary for the fusion of the myeloma cell line Sp2/0-Ag 14 (Sp2/0) to mouse spleen cells and the subsequent production of high titer monoclonal antibodies.

Immunization

The preparation of and immunization with *E. coli* somatic antigens (OAgS), *E. coli* β-galactosidase, and *E. coli* β-glucuronidase are described in the text. It is important that the mouse be "stimulated" immunologically to the antigen of interest for successful fusions. Therefore, it is important that several immunization protocols be tested and the response to the antigen identified prior to fusion. Mouse serum which produced a positive reaction by EIA indicated that the mice had been successfully immunized.

The form or state of the antigen is also important. Because monoclonal antibodies are monospecific, the state of the antigen injected must be the same as the state of the antigen for which the resulting monoclonal antibodies will be used to identify. For example, monoclonal antibodies produced to a non-denatured protein in many instances will not react with denatured protein in Western blots. Therefore, it is important to know how and in what kind of assay the monoclonal antibodies
will be used and the state of the antigen prior to immunization for the production of monoclonal antibodies.

**Growth and maintenance of the Sp2/0 and hybridoma cell lines**

The myeloma cell line Sp2/0 was obtained from R. Diaco (Dept. of Microbiology, Iowa State University, Ames, IA). The Sp2/0 cell line does not produce immunoglobulin and it lacks the enzyme hypoxanthine-guanine phosphoribosyltransferase (HGPRT) which makes it susceptible to aminopterin. The Sp2/0 cells can be grown in several culture media but Iscove's modified Dulbecco's medium (IMDM) was used because the HEPES buffer maintained the pH of the medium near neutrality when outside of the CO₂ incubator. This was crucial when spleen and fused cells were in small volumes of medium during the fusion procedure. Dulbecco's modified Eagle medium became alkaline when outside of the CO₂ incubator. The pH of the growth medium must be kept near neutrality because the Sp2/0 cell line is susceptible to alkaline conditions; this was also important when cloning or feeding newly formed hybrids. The source (Sigma or Gibco) of the medium constituents and other reagents did not seem to affect the growth of the Sp2/0 cells or hybridomas. The composition of IMDM was:

<table>
<thead>
<tr>
<th>Component</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Powdered IMDM (Sigma #I-7633)</td>
<td>10.0 g</td>
</tr>
<tr>
<td>2 mercaptoethanol (100x = 70 ul/l)</td>
<td>10.0 ml</td>
</tr>
<tr>
<td>Sodium bicarbonate (Sigma #S-5761)</td>
<td>3.0 g</td>
</tr>
<tr>
<td>Fetal bovine serum (Hazelton Dutchland, Inc.,  #12-10378)</td>
<td>200.0 ml</td>
</tr>
<tr>
<td>Deionized water (resistivity 18-megohm/cm)</td>
<td>800.0 ml</td>
</tr>
</tbody>
</table>
The final pH was between 7.2 and 7.4 and usually did not require adjusting. The medium was filtered through a 0.2-um filter, 90 mm in diameter (Sewler Industries Inc., Franklin Park, IL; #NM 14201-020SP). When quantities of IMDM greater than 3 liters were to be sterilized, the medium was prefiltered through a 0.2-um filter, 142 mm in diameter (Sewler Industries Inc., Franklin Park, IL; #NM 090-01-020SP) prior to passage through the 90-mm filter. The sterile IMDM was bottled aseptically in 100-ml and 500-ml aliquots and stored at -20°C. For use, the media were thawed and were stored at 4°C for up to 2 weeks.

8-azaguanine (8-aza) was used to ensure that the Sp2/0 cell line was HPGRT\(^{-}\) and sensitive to aminopterin. Because 8-aza dissolves slowly in water, 2.0 mg of 8-aza (Sigma #A-8526) was added to 1 liter of deionized water and incubated overnight at 37°C with stirring. IMDM-8-aza was prepared by using 8-aza solution instead of deionized water.

IMDM-8-aza was used to culture the Sp2/0 cells, except that IMDM was used during the week preceding a fusion and when cells were being grown to harvest conditioned medium. IMDM-CM was used for newly formed hybrids and when cloning (see below). Sp2/0 cells and cloned hybridomas were grown in 25-cm\(^2\) tissue culture flasks (Bellco #4232-03050) incubated at 37°C with 5% CO\(_2\) in a humidified CO\(_2\) incubator. The flasks were incubated on their sides with the caps slightly loose. The Sp2/0 cells, when growing properly, required transfer every 2 to 3 days, as indicated by cell density and the change of the phenol red pH indicator from red to yellow-orange. The cells were diluted 1:20 when transferred. Log phase Sp2/0 cells were required for successful fusions; these appeared
as round, smooth, refractive cells when viewed with a phase-contrast microscope.

Feeder cell-layers are often used to grow newly fused cells and to clone resulting hybridomas. The feeder layers are thought to supply an essential nutrient or stimulatory product(s) which enhances the growth of the hybridomas. Because of the difficulties associated with culturing hybridomas with feeder layers, medium containing the byproducts from the growth of feeder cells (conditioned medium) is often used to supplement growth media. Conditioned medium from mouse peritoneal macrophages was usually used 1:1 with IMDM (IMDM-CM) for the growth of newly formed hybridomas and when cloning. Alternatively, conditioned medium from the Sp2/0 cell line themselves was used in place of macrophage-conditioned medium. Whether macrophage- or Sp2/0-conditioned medium was used, the cells were allowed to grow for 2 to 3 days or until the medium changed to a yellow-orange color. The cells were then pelleted by centrifugation and the supernatant mixed with IMDM. Conditioned medium could be stored at -20°C if not used immediately.

Cell fusion

To ensure that the Sp2/0 cells were healthy and in the proper stage of growth, 0.1 ml of the cell suspension was removed and added to a tube containing 0.9 ml of an aqueous solution of trypan blue (Allied Chemical, New York, NY; #508). The stain was prepared by diluting a 4% stock solution 1:50 with 0.01 M phosphate buffered saline (KH$_2$PO$_4$, 0.2 g; Na$_2$HPO$_4$, 1.15 g; NaCl, 8.5 g; distilled H$_2$O, 1000 ml; pH 7.4). A small portion
of the diluted Sp2/0 cell suspension was transferred to a hemacytometer and the viable (nonstaining) cells were enumerated; nonviable cells and debris stained blue. When diluted 1:20, healthy Sp2/0 cells reached \(10^5\) cells/ml in 48 h. Since \(3-5 \times 10^7\) Sp2/0 cells were needed for a fusion, from 30 to 50 ml of Sp2/0 cells were required.

An immunized mouse was placed in a beaker and CO\(_2\) was added until the mouse was unconscious. The mouse was then bled by cardiac puncture. The blood was allowed to clot overnight at 4\(^\circ\)C and the serum collected. The serum was used in subsequent EIA as a positive control and to confirm that the mouse had been stimulated by the antigen injected. The mouse was then euthanized by CO\(_2\) asphyxiation. The mouse was disinfected by submerging in a beaker containing 70\% ethanol and then was transferred to a laminar flow hood. Using sterile scissors and forceps, the skin was cut, starting at the lower left back and proceeding across the abdomen to the right side of the mouse being careful not to cut the peritoneal-lining. The skin was peeled back by grabbing the skin on both sides of the incision and pulling each half towards the head and tail simultaneously. The spleen was partially visible under the left ribs. With a fresh pair of sterile scissors and forceps, the peritoneal-lining was cut well below the spleen so as not to cut it. A circular incision was made around the spleen, while using forceps to pull the flap of skin away from the incision. The spleen appeared as a lobular dark purple organ just below and partially covered by the lower left ribs; however, in some mice it was covered with fat. Another set of sterile scissors
and forceps was used to remove the spleen. The spleen was held with
the forceps and was pulled from the cavity while using the scissors to
cut the connective tissue. The spleen was placed in a sterile 60 x 15
mm tissue culture dish (Fisher #08-772-21) containing 5.0 ml of IMDM.
The spleen was perfused 5 times by using a 1-ml syringe with a 26 gauge
needle. Each perfusion consisted of 1.0 ml of IMDM obtained from the
tissue culture plate containing the spleen. The remainder of the spleen
was discarded. After gently but thoroughly mixing the cell suspension,
the spleen cells were enumerated by using trypan blue stain as described
for the Sp2/0 cells except that a second 1:10 dilution of the spleen
cells was used for counting (final dilution 1:100). Only nonstaining
round cells were counted. Spleens from mice which were properly immunized
yielded 1 x 10^8 cells/ml or greater.

After determining the number of viable spleen cells present, enough
cells were added to a sterile 50-ml polypropylene centrifuge tube (Fisher
#05-538-55) containing 30-50 ml of Sp2/0 cells (3-5 x 10^7 Sp2/0 cells
total) to achieve a ratio of at least 2 spleen cells to 1 Sp2/0 cell.
After centrifugation of the mixed-cell suspension at 200 x g for 8
minutes, the supernatant was decanted into a beaker and saved. The pellet
was gently resuspended in the remaining supernatant by tapping the end
of the tube.

Although successful fusions were carried out by using polyethylene
glycol (PEG) from other sources, fusions with PEG 1500 (BMB #779 512)
produced the most hybrids. Evidently the buffering of the PEG solution
with Hepes and the elimination of aldehydes and peroxides following auto-
claving reduces the toxicity of the PEG. To the resuspended pellet, 1.0 ml of PEG was added gradually over a period of 15 seconds. The mixture was swirled for 30 seconds and placed in a beaker containing water at 37°C for 90 seconds. Then 1.0 ml of IMDM was added over 15 seconds, and the contents were mixed and incubated at 37°C for an additional 30 seconds. Finally, 10 ml of IMDM was added during a period of 1 minute, after which the tube was incubated at 37°C for 5 minutes.

Following incubation, the cells were pelleted by centrifugation at 200 x g for 8 minutes. The supernatant was discarded making sure that practically all of the PEG-containing supernatant was removed as possible. Based upon the number of Sp2/0 cells present, the pellet was resuspended in enough IMDM-CM-HAT to obtain a final concentration of 5 x 10⁵ cells/ml. Prior to adding the entire volume of IMDM-CM-HAT, a small portion was added with a broad-bore pipette to gently resuspend the pellet. IMDM-CM-HAT was prepared by adding the macrophage or Sp2/0 conditioned medium, which was collected earlier, to an equal volume of IMDM and adding sufficient 100x hypoxanthine-aminopterin-thymidine (Hazelton-Dutchland Inc., #59-77076) to obtain a 1x HAT medium. The cell suspension in IMDM-CM-HAT was dispensed in volumes of 0.1 to 0.2 ml (2 drops)/well of a 96-well tissue culture plate (Bellco #4250-03596). The plates were incubated at 37°C under 5% CO₂ in a humidified CO₂ incubator.

Because the myeloma cells are HPGRT−, the aminopterin present in IMDM-CM-HAT prevents the growth of unfused Sp2/0 cells. Unfused spleen cells survive for only a few days in tissue culture. Therefore, the
only cells which will grow in IMDM-CM-HAT are cells resulting from the fusion of a spleen cell which provides a functional HPGRT and a myeloma cell which provides immortality to the cell line.

Following fusion, the cells were fed every 3-4 days (earlier if the growth medium turned yellow) by adding 2 drops of IMDM-CM-HAT to each well. If the growth medium was still yellow after the addition of 2 drops of IMDM-CM-HAT, additional medium was added until the pH was near neutrality (the medium was reddish-orange in color). In subsequent feedings, spent medium was removed from the wells by aspiration and was replaced with fresh IMDM-CM-HAT. By the fifth day following fusion, hybridomas were visible as clusters of refractive cells when examined at 10x magnification with an inverted phase-contrast microscope. The hybrids were assayed for antibody production by using an indirect EIA approximately 10 days after fusing or when the culture had been fed for the third time.

Cloning and expansion of antibody-secreting hybridomas

Antibody-secreting hybridomas were cloned to ensure that each antibody-secreting cell line was free of nonantibody producing cells which might outgrow it. A modification of the limiting-dilution method described by Galfre and Milstein (1981) was used to clone the hybridomas. The progressive "scaling-up" of hybridomas from one well of a 96-well tissue culture plate to 2 wells of a 96-well plate to a single well of a 24-well plate (Bellco or Fischer #08-757-156) and finally into a 25-cm$^2$ tissue culture flask prior to cloning takes 10-15 days to complete.
These steps were bypassed. Cloning was performed directly from the 96-well plate. The cloning of several hybrids consistently yielded between $5.0 \times 10^5$ and $1.0 \times 10^6$ hybridomas/ml in a single well of 96-well plate. Therefore, enumerating the hybridoma cells was not necessary. Hybridomas secreting antibodies with the desired specificities were thoroughly resuspended by using a cotton-plugged 2.0 ml pipette and Pasteur pipette bulb. One drop (approximately 50 ul) of the cell suspension was added to a sterile 13 x 100 mm tube containing 5.0 ml of IMDM-CM-HT (1x hypoxanthine-thymidine; $10^{-2}$ dilution) and the remainder was added to the well of a 24-well plate containing 1.0 ml of IMDM-CM-HT. The tube was inverted several times and then 1 drop was transferred to a second tube containing 5.0 ml of IMDM-CM-HT ($10^{-4}$ dilution). The tube was inverted several times and the contents of the tube were dispensed into 96-well tissue culture plates at 0.1 ml/well. (Originally, another $10^{-1}$ dilution was made and the contents of the last two tubes were dispensed; however, the contents of the last tube consistently failed to yield hybrids. Therefore, the extra dilution was eliminated from the direct-cloning procedure). The plates were incubated in a humidified incubator under 5% CO$_2$ at 37°C. Cells in the 24-well plate were fed as needed with IMDM-CM-HT until growth was confluent (3-5 days) and then the contents were frozen as described later.

IMDM-CM-HT was prepared by adding sufficient 100x hypoxanthine-thymidine (HT; Hazelton-Dutchland #59-57076) to IMDM-CM to result in 1x IMDM-CM-HT. IMDM-CM-HT was required for growth of the hybridomas until aminopterin had been diluted out by cloning and subsequent feedings (2-3 feedings with IMDM-CM-HT was sufficient).
Seven to 10 days after cloning, the wells were examined either visually or at 10x magnification with an inverted phase-contrast microscope for the presence of single hybridoma colonies. The positive wells were fed with 0.1 ml of IMDM-CM-HT, and prior to the next feeding (usually after 3 to 4 days) were assayed by EIA for the presence of antibody. Wells containing a single antibody-secreting clone were thoroughly resuspended and cloned as described previously. The cloning cycle was generally repeated three times or until 100% of the wells containing hybrids were positive for antibody production. If cloning failed to produce antibody-secreting clones, cells from the 24-well plate which had been frozen were thawed and cloned in IMDM-CM-HT. After cloning was complete, the hybridomas were expanded and frozen as soon as possible.

Prior to freezing and injecting into mice for production of ascites fluid, the cloned hybridomas were expanded from one well of a 96-well plate into a single well of a 24-well plate containing 1.0 ml of IMDM. The cells were fed every 3-4 days (earlier if the medium turned yellow) by aspirating off one-half of the growth medium and replacing it with enough fresh IMDM to bring the pH to near neutrality (orange-red color). When the cells covered the bottom of the well, the cells were resuspended and transferred to a 25-cm$^2$ flask containing 5.0 ml of IMDM. After feeding with an additional 5.0 ml of IMDM (usually after 3 days), subsequent feedings were made by transferring 1.0 ml into a 25-cm$^2$ flask containing 9.0 ml of IMDM. The remaining cells were frozen and injected into mice for production of ascites fluid.
Cell freezing and thawing

Sp2/0 cells and cloned hybridomas to be frozen were grown in 25-cm$^2$ flasks on their sides with the caps loose until the medium turned an orange-yellow color. The cell densities were approximately $5 \times 10^5$ cells/ml. Ten milliliters of the cell suspension were transferred to a 15-ml conical centrifuge tube and the cells were pelleted by centrifugation at 225 x g for 10 minutes. The pellet was gently resuspended in 5.0 ml of freezing medium and 1.0-ml portions were dispensed into 2.0 ml Cryotubes (Nunc #368632). The freezing medium consisted of 10% dimethyl sulfoxide (Sigma #D-2650) in fetal bovine serum. Cells from 24-well plates were frozen by removing all of the growth medium and resuspending the cells in 1.0 ml of freezing medium. The Cryotubes were placed in a Nunc transport container (Vangard #241) which was placed into a freezing chamber which encompassed the transport container on all sides by approximately 1 inch of styrofoam. The freezing chamber was placed into a -70°C cryogenic freezer for 24 h. On the next day, the transport container was removed from the freezing chamber and returned to the -70°C freezer. The cells could be stored in this manner for 6-9 months.

When thawing frozen hybridomas, the Cryotubes were removed from the freezer and placed at 37°C until thawed. Each tube was inverted several times and the contents transferred to a sterile 15-ml conical centrifuge tube containing 10.0 ml of IMDM. The cells were pelleted by centrifugation at 225 x g for 10 minutes, resuspended in 2.0 ml of IMDM-CM, dispensed into 2 wells of a 24-well plate, and incubated in
a humidified incubator under 5% CO₂ at 37°C. Feeding with IMDM-CM was carried out as required with IMDM-CM until growth covered the bottom of the well. At this time the cells were transferred to flasks and fed with IMDM as needed.

**Ascites fluid production**

High titered ascites fluid was produced in mice which had been injected 10-14 days earlier with 0.5 ml of 2,6,10,14 tetramethylpentadecane (Pristane, Sigma #T-7640). Ten-milliliters of an antibody-producing hybridoma at a density of approximately 5.0 x 10⁵ cells/ml were pelleted by centrifugation at 225 x g for 10 minutes and resuspended in 1.0 ml of PBS prewarmed to 37°C. Three mice were injected intraperitoneally, each with 0.3 ml of the cell suspension.

The mice were observed daily for abdominal swelling. Usually 6-9 days after injection, the abdomen became extended due to the production of ascites fluid. The fluid was removed by inserting an 18-gauge needle into the peritoneal cavity. The fluid was collected in a sterile conical centrifuge tube and cells and debris removed by centrifugation at 1000 x g for 10 minutes. The mice were drained again 2-5 days later and then were sacrificed. Generally, 10-15 ml of ascites fluid could be collected from a single mouse. The ascites fluid was split into 2.0-ml aliquots and was stored at -20°C.

For additional information on monoclonal antibody production see Fasekas de St. Groth and Scheidegger (1980) and Van Deusen and Whetstone (1981).


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