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Abstract
Eleven bacterial and two yeast strains, four of which were previously identified as having activity on a lightly cross-linked carboxymethyl cellulose (CLD-2) found in one type of superabsorbent tampon, were grown on a variety of substrates, most containing cellulosics. None produced detectable amounts of cellulases, but all elaborated beta-glucosidase. None of these 13 strains nor 3 commercially obtained beta-glucosidase preparations could hydrolyze CLD-2, although a commercial cellulase and two other bacterial preparations known to produce cellulases could. Based on these results, it appears that previous work suggesting that the degradation of CLD-2 by vaginal microbes and beta-glucosidase is implicated in the production by Staphylococcus aureus of toxin causing toxic shock syndrome must be reevaluated.

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Application of Cross-Linked Carboxymethyl Cellulose Degradation by β-Glucosidase and Vaginal Microbes to Toxic Shock Syndrome

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Eleven bacterial and two yeast strains, four of which were previously identified as having activity on a lightly cross-linked carboxymethyl cellulose (CLD-2) found in one type of superabsorbent tampon, were grown on a variety of substrates, most containing cellulosics. None produced detectable amounts of cellulases, but all elaborated β-glucosidase. None of these 13 strains nor 3 commercially obtained β-glucosidase preparations could hydrolyze CLD-2, although a commercial cellulase and two other bacterial preparations known to produce cellulases could. Based on these results, it appears that previous work suggesting that the degradation of CLD-2 by vaginal microbes and β-glucosidase is implicated in the production by *Staphylococcus aureus* of toxin causing toxic shock syndrome must be reevaluated.

Toxic shock syndrome (TSS) was originally described and linked to the presence of *Staphylococcus aureus* by Todd et al. (32) in 1978. The incidence of TSS has been linked by statistical inference to the use of tampons, especially those of high absorbency, such as Procter & Gamble's Rely brand (4–7, 11, 22, 27). Up to this point there has been no generally accepted explanation for the association between tampons and TSS.

Recently, Tierno (30) and Hanna (10) suggested that one of the absorbents in Rely tampons plays a role in the occurrence of TSS. Rely tampons contain a lightly cross-linked carboxymethyl cellulose (CLD-2) in the form of fibers and present in the tampon as small chips. According to them, the CLD-2 fibers can be enzymatically degraded by microbes found in the vagina during menstruation, the products of the degradation encouraging the growth of *S. aureus* and its subsequent toxin production. Tierno (30) estimated the enzymatic degradation of the CLD-2 fibers by checking for an increase in the mobility of a concentrated gellike aqueous suspension, and Hanna (10) used the same method and in addition tested for the appearance of reducing sugars released during hydrolysis by using the method of Mandels et al. (16). Tierno et al. (31), using commercially obtained enzyme samples in assays that were similar to those used previously (10, 30), claimed that a particular enzyme, β-glucosidase, was capable of degrading the CLD-2 fibers. As virtually all of the organisms they used are known to inhabit the vagina (1, 13–15, 17, 20, 21, 26, 29) and, as many in addition have been reported to produce β-glucosidase (3, 12), they concluded that CLD-2 could serve as an external source of nutrients for *S. aureus* in vivo (31).

These results raised some questions, as it was not expected that β-glucosidase by itself would be capable of attacking long-chain cellulosic molecules (23, 33) such as those found in CLD-2. In addition, an increase in the mobility of a CLD-2 fiber suspension may not be due to enzymatic degradation but rather to fiber dehydration caused by an increase in ionic strength (9). Furthermore, the dinitrosalicylic acid technique used by Hanna (10) and Tierno et al.(31) to measure reducing sugars has been shown to be inaccurate with the complex substrates used (24, 25). Therefore, we studied the cellulolytic activity of vaginal bacteria and yeasts more extensively. A number of cultures used by Tierno (30) in his studies were grown in several different media with various substrates to induce cellulolytic enzyme production. Commercially obtained samples of β-glucosidase and cellulase were used to test for activity on CLD-2. Changes in the ionic strength of CLD-2 fiber suspensions after the addition of small amounts of bacterial cultures were also studied, as this could provide an alternate explanation for the mobility results obtained by Tierno (30) and Hanna (10).

MATERIALS AND METHODS

Cultures. One culture from each of 13 species (11 bacteria and 2 yeasts) was received from Procter & Gamble, which received it from Tierno, supposedly from those he had tested (30). Of the species sent to us, four had at least some cultures that were reported by Tierno to increase the mobility of gellike suspensions formed by CLD-2 in water: *Klebsiella oxytoca* (two positive, zero negative cultures reported by him), *Klebsiella pneumoniae* (four positive, one negative), *Proteus mirabilis* (one positive, one negative), and *Serratia marcescens* (two positive, zero negative). Therefore, two of the cultures sent to us, representatives of *K. oxytoca* and *S. marcescens*, surely had activity in Tierno's experiments. He reported that the other nine species we received, *Acinetobacter anitratus*, *Bacillus subtilis*, *Candida albicans*, *S. aureus*, *Staphylococcus epidermidis*, *Streptococcus agalactiae*, *Streptococcus faecium*, *Streptococcus* group G sp., and *Torulopsis glabrata*, had no cultures with activity. Hanna (10) tested six of these species, *K. oxytoca*, *K. pneumoniae*, *P. mirabilis*, *S. marcescens*, *S. aureus*, and *S. epidermidis*, though not necessarily the same cultures tested by Tierno, and found, like Tierno, that the first four had some cultures that could increase mobility or reducing sugars or both in CLD-2 suspensions.

Two bacterial cultures, *Cellulomonas uda* NRRL B-404 and *Cellvibrio gilvus* NRRL B-14078, were received from the Northern Regional Research Center, Peoria, Ill. These were used as controls for cellulase production, as both have been reported to have cellulase activity (2, 18).

Growth tests. To promote the production of β-glucosidase and cellulases, we grew all 13 Tierno cultures in 3% Trypticase soy broth (catalog no. 11768, lot E3DKL1; BBL Microbiology Systems) alone or with one of six different substrates

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added. The latter included lactose, cellobiose, amorphous cellulose (catalog no. 286; Schleicher & Schuell, Inc.), crystalline cellulose (catalog no. 144; Schleicher & Schuell), carboxymethyl cellulose (catalog no. C-8758, lot 42F-0438, low viscosity; Sigma Chemical Co.) (all 0.25% [wt/vol]), and 0.125% (wt/vol) CLD-2 chips (lot 9109BD; degree of substitution, between 0.63 and 0.92 per unit of glucose; Buckeye Cellulose Corp.). The two control cultures were grown in Trypticase soy broth alone and with either carboxymethyl cellulose or CLD-2 (0.25 and 0.125%, respectively) added. All cultures were also grown in a simulated Trypticase soy broth solution (composed of its individual components without added glucose) alone and with CLD-2 (0.125%) as a substrate and also in 2.5% heart infusion broth (catalog no. 0038-01, control 653040; Difco Laboratories) with CLD-2 (0.125%) as a substrate.

All growth studies were conducted in 50 ml of liquid contained in 250-ml Erlenmeyer flasks closed with polyurethane sponges and subjected to rotary shaking (100 rpm, Environ-Shaker 3597, Lap-Line Instruments, Inc.) at 37°C. Autoclaving was done at 121°C for 15 min. Stock and working cultures were carried on 3% Trypticase soy agar slants, and inoculation of liquid cultures was done with a platinum loop. All cultures were grown to the stationary phase, as determined by optical density. At that point glucose, in those flasks originally containing it, was below 0.006%.

Enzyme assays. All enzyme assays were carried out on centrifuged broth samples as well as on sonicated cell samples. Centrifuged cells, suspended to the original volume in phosphate buffer, were sonicated with a Heat Systems model W-375 cell disrupter until at least a 20% decrease in optical density occurred (40% was the average loss) to release intracellular enzymes. Afterwards, 200 μl of each enzyme sample was incubated for 1 h at 37°C with 1 ml of α-cellulose (catalog no. C-8002, lot 22F-0540; Sigma), carboxymethyl cellulose, or CLD-2 (all 0.1%) in 0.05 M sodium phosphate buffer (pH 7.3). Cellulase and carboxymethyl cellulase activities were assayed by the Somogyi-Nelson method (19, 28). The detection limit of these assays was 0.01 IU/ml.

β-Glucosidase activity was determined by two methods. The first used *p*-nitrophenyl-β-D-glucopyranoside as a substrate. A 1-ml enzyme sample was added to 1 ml of 4 mM *p*-nitrophenyl-β-D-glucopyranoside in phosphate buffer and incubated at 37°C for 1, 2, or 3 h. The reaction was stopped by the addition of 2 ml of 20% Na_2CO_3. Activity was measured as the absorbance of released *p*-nitrophenol at 400 nm. The detection limit was 9×10^{-6} IU/ml. The second method used cellobiose as a substrate and tested for glucose production with a Beckman model ERA-1002 glucose analyzer. A 1-ml enzyme sample was added to 1 ml of 1% cellobiose in phosphate buffer and incubated at 37°C for 1, 2, or 3 h. The detection limit was 1.9×10^{-3} IU/ml. The two β-glucosidase assays were performed on samples from the following five growth substrates only : Trypticase soy broth with lactose, cellobiose, or CLD-2 added, the simulated soy broth alone, and the simulated soy broth with CLD-2 added.

Enzymatic digestion of CLD-2. Three β-glucosidase preparations, catalog no. G-8625 (lot 33F-4003, 6.3 IU/mg; Sigma), catalog no. G-4511 (lot 122F-4004, 38 IU/mg; Sigma), and catalog no. 15505 (lot 41274, 5 IU/mg; United States Biochemical Corp.), and one cellulase preparation, catalog no. C-2274 (lot 122F-0906, 2 IU/mg; Sigma), were used to study the digestion of CLD-2. Digestion with the β-glucosidases was carried out in a 1% CLD-2 suspension in phosphate buffer and in sodium citrate buffer (pH 5.5, 0.05 M) for 48 h at 37°C. In all cases, an enzyme concentration of 0.1 IU/ml was used. The digestion of CLD-2 by cellulase was carried out in phosphate buffer with an enzyme concentration of 1 IU/ml for 24 h at 37°C.

Detection of carbohydrates from enzymatic digestion of CLD-2. High-pressure liquid chromatography of samples from the enzymatic digestion was done with a Waters model 6000A solvent system, model U6K injector, and model R401 refractive-index detector. The column was a Bio-Rad HPX-42A column with a water eluent at 85°C and 0.4 ml/min. The detection limit for each peak was 2 μg (1 mM for glucose).

Glucose concentrations in the CLD-2 digests were determined by a modification of the glucose oxidase method of Fleming and Pegler (8) by adding 1 ml of glucose oxidase reagent (pH 7.0) to 0.4 ml of sample and incubating the mixture at 35°C for 1 h. Afterwards, 2 ml of 7 N HCl was added to stop enzymatic activity. Glucose concentrations were determined by measuring the absorbances at 525 nm. The detection limit was 0.005 mM.

Ionic strength determination. The ionic strength of suspensions containing CLD-2 was determined indirectly by measuring their conductivity with a Hach model 2510 conductivity meter. The conductivities of 50 ml of 1 or 2% suspensions of CLD-2 in deionized water were determined before and after the addition of 0.5 ml of culture broth containing *K. oxytoca*, *K. pneumoniae*, *P. mirabilis*, or *S. marcescens*. Samples of 10 ml of Trypticase soy broth were inoculated with a loopful of each culture and incubated for 24 h at 37°C before being added to the CLD-2 suspensions.

RESULTS

The results of the assays for cellulolytic activity based on reducing sugar values are shown in Table 1. Of the 13 Tierno cultures grown in each of the 10 growth substrates, none had detectable cellulase or carboxymethyl cellulase activity, either intracellular or extracellular, in any of the three assays with α-cellulose, carboxymethyl cellulose, and CLD-2 as substrates. The two control cultures, *C. uda* and *C. gilvus*, had positive cellulase and carboxymethyl cellulase activities

TABLE 1. Maximum cellulase activity of centrifuged broth samples or sonicated cell samples after growth of selected vaginal microorganisms

Microorganism	Cellulase activity (IU/ml) on[a]:		
	α-Cellulose	Carboxymethyl cellulose	CLD-2
A. anitratus	—	—	—
B. subtilis	—	—	—
C. albicans	—	—	—
K. oxytoca	—	—	—
K. pneumoniae	—	—	—
P. mirabilis	—	—	—
S. marcescens	—	—	—
S. aureus	—	—	—
S. epidermidis	—	—	—
S. agalactiae	—	—	—
S. faecium	—	—	—
Streptococcus group G sp.	—	—	—
T. glabrata	—	—	—
C. uda (control)	0.01	0.05	0.06
C. gilvus (control)	0.03	0.03	0.08

[a] —, Activity was below the detection limit (0.01 IU/ml) for all growth substrates tested.

TABLE 2. Maximum β-glucosidase activity of centrifuged broth samples and sonicated cell samples after growth of selected vaginal microorganisms

Microorganism	β-Glucosidase activity (10^3) (IU/ml) on[a]:			
	p-Nitrophenyl-β-D-glucopyranoside[b]		Cellobiose[c]	
	Centrifuged	Sonicated	Centrifuged	Sonicated
A. anitratus	0.36	0.08	—	—
B. subtilis	—	0.40	—	3.1
C. albicans	3.10	1.80	3.7	4.3
K. oxytoca	0.09	0.76	—	3.7
K. pneumoniae	—	0.26	—	2.5
P. mirabilis	—	0.16	—	—
S. marcescens	0.76	0.19	—	3.1
S. aureus	0.52	0.05	4.3	—
S. epidermidis	7.90	1.20	5.6	5.0
S. agalactiae	0.09	—	—	—
S. faecium	0.04	0.02	—	—
Streptococcus group G sp.	—	0.10	—	—
T. glabrata	0.04	0.10	—	—
C. uda (control)	—	6.70	NT[d]	8.6
C. gilvus (control)	0.59	4.90	NT	5.6

[a] —, Activity was below the detection limit for all growth substrates tested.
[b] Detection limit, 0.009×10^{-3} IU/ml.
[c] Detection limit, 1.9×10^{-3} IU/ml.
[d] NT, Not tested.

in all three assays when grown in each of the six growth substrates used for the controls. The highest values attained with any of the growth substrates are shown in Table 1.

The results of the β-glucosidase assays (Table 2) indicated that all 13 Tierno cultures, as well as the two control cultures, had β-glucosidase activity on *p*-nitrophenyl-β-D-glucopyranoside with at least one of the growth substrates. The two control cultures and seven of the other cultures had β-glucosidase activity in the less sensitive assay with cellobiose as the assay substrate. Generally, intracellular activity was higher than extracellular activity.

The digestion of CLD-2 with each of the three commercially obtained β-glucosidase samples did not lead to any production of glucose in the glucose oxidase assay or any production of glucose or lower oligosaccharides in the high-pressure liquid chromatography analysis. The digestion of CLD-2 with a commercially obtained cellulase sample released 0.19 mM glucose after 24 h in the glucose oxidase assay and yielded three peaks in the high-pressure liquid chromatography analysis: glucose, cellobiose, and a peak which is most likely a carboxylated cellooligosaccharide. The third peak did not appear when the same sample was analyzed by high-pressure liquid chromatography with an amine-bonded silica column, which would bind to and therefore not release a charged carboxylated compound.

The addition of culture broth to a 1 or 2% suspension of CLD-2 resulted in an increase in conductivity with each of the four cultures tested (Table 3).

DISCUSSION

None of the 13 Tierno cultures had cellulase activity with any of the 10 different substrates used for growth. Three different assays were used to test for general cellulase activity or for specific activity on the substrates carboxymethyl cellulose and CLD-2. However, all of the cultures tested had β-glucosidase activity. Tierno (30) and Hanna (10) found an increase in the mobility of concentrated CLD-2 suspensions in only 4 of the 13 species tested here, indicating that an increase in mobility is not a good indicator of the ability of microbial cells to form either cellulase or β-glucosidases.

As noted previously, β-glucosidase by itself would not be expected to degrade long-chain cellulosic materials, particularly those with side chains like those present in CLD-2 (23). The results of CLD-2 digestion with three commercially obtained β-glucosidase samples did not indicate any hydrolysis of CLD-2 to glucose or lower oligosaccharides, although the presence of these components was readily detectable when the hydrolysis of CLD-2 was carried out with a commercially obtained cellulase sample. It is therefore clear that β-glucosidase cannot hydrolyze CLD-2 without the additional presence of a cellulase. As there was no detectable cellulase activity present in the cultures tested and as the β-glucosidase found in the growth studies had less than 10% the activity of that used in the digestion studies, it should not be expected that the native microbial β-glucosidases studied here could hydrolyze CLD-2 to glucose. Our studies revealed that they could not. Similar

TABLE 3. CLD-2 suspension conductivities before and after the addition of microbial culture broth

Microorganism	% CLD-2	Conductivity (μS/cm)	
		Before broth addition	After broth addition
K. oxytoca	1	620	800
	2	1,400	1,600
K. pneumoniae	1	600	850
	2	1,400	1,650
P. mirabilis	1	600	820
	2	1,450	1,750
S. marcescens	1	600	810
	2	1,450	1,700

results with a different approach were also noted by E. T. Reese and M. Mandels (personal communication). Using three β-glucosidase preparations from almonds, they found no detectable activity on carboxymethyl cellulose. Using a fungal β-glucosidase preparation, they detected hydrolysis of carboxymethyl cellulose. This hydrolysis was attributed to trace amounts of a random-acting cellulase and not to β-glucosidase, as nojirimycin, a strong inhibitor of β-glucosidase, had no effect on hydrolysis in subsequent studies.

Of the 13 Tierno cultures, *K. pneumoniae*, *K. oxytoca*, *P. mirabilis*, and *S. marcescens* were reported to have activity on CLD-2 based on suspension mobility studies (30). Hanna (10) also reported activity based on mobility tests for the first two of these cultures but not the last two. All four of these cultures produced an increase in conductivity when added to CLD-2 solutions. An increase in conductivity and therefore ionic strength causes CLD-2 fibers to desorb water (9), which can cause an increase in the mobility of CLD-2 suspensions. As the native β-glucosidases of these cultures do not hydrolyze CLD-2, the increase in suspension mobility observed with these cultures could possibly be attributed to an increase in ionic strength but not to enzymatic hydrolysis of the fibers by β-glucosidase.

The role of superabsorbent tampons, including Rely, in TSS is not clear. It is apparent that the absorbent CLD-2 present in Rely tampons is not degraded by the microbes tested and therefore does not provide nutrients for the growth of *S. aureus*.

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ADDENDUM IN PROOF

While this article was awaiting publication, a note (J. T. Mills, J. Parsonnet, Y.-C. Tsai, M. Kendrick, R. K. Hickman, and E. H. Kass, J. Infect. Dis. **151**:1158–1161, 1985) appeared which stated that the polyester foam, but not the carboxymethyl cellulose, in Rely tampons, adsorbed Mg^{2+} and that this stimulated toxin production.

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