Solubilization of the Conjugation Inhibitor from *Escherichia coli* Cell Wall

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ABSTRACT

SCHWARTZ, GABRIEL H. (National Institute of Arthritis and Metabolic Diseases, Bethesda, Md.), DONALD EILER, AND MILTON KERN. Solubilization of the conjugation inhibitor from *Escherichia coli* cell wall. J. Bacteriol. 89:89-94. 1965.—Conjugation between F⁻ and HfrC strains of *Escherichia coli* is inhibited by the presence of cell wall derived from either strain. The inhibitor can be solubilized from such cell walls or whole cells by treatment with periodate. The soluble material is relatively acid-labile and alkali-stable, nondialyzable, nonsedimentable at 100,000 × *g* (1 hr), partially sedimented after 3 hr at 100,000 × *g*, sensitive to small changes in ionic strength, neutralized by cationic reagents, and is insensitive to proteinases, nucleases, lipases, and carbohydrases. The soluble inhibitor derived from male and female cells is indistinguishable by all the criteria tested.

During sexual reproduction of *Escherichia coli*, the initial effective contact between male and female cells most likely involves the interaction between complementary structures. Such cell interactions are vital as a prelude to the ultimate transfer of the genetic matter and may involve processes fundamentally similar to those underlying the cell-cell interactions occurring in aggregation and tissue differentiation (Steinberg, 1963).

A convenient assay for the interaction of the "sexual receptor sites" based on the specific inhibition of conjugation by cell wall was described by Kern (1962) and Yura (1962), and was recently confirmed by Lancaster, Goldschmidt, and Wyss (1964). This report is concerned with the solubilization and attempts at characterization of the conjugation inhibitor.

MATERIALS AND METHODS

The bacterial strains employed included *E. coli* C600B (F⁻, *thr*⁻, *leu*⁻, B₁⁻, and streptomycin-resistant), *E. coli* 1895 (male HfrC, *meth*⁻, streptomycin-sensitive), *Alcaligenes faecalis*, *Staphylococcus aureus*, and *Micrococcus lysodeikticus*. The bacteria were grown on Difco Antibiotic Media #3, and large lots were harvested by Sharples centrifugation and stored at -15 C.

The conjugation assay was performed by add-

ing 0.25 ml each of log-phase suspensions of strains 1895 and C600B to 0.5 ml of 0.01 M phosphate (pH 7.0). Where indicated, the 0.5 ml of buffer was replaced by the inhibitor or other additions in an equal volume of the same phosphate buffer. The mating mixture was incubated for 1 hr at 37 C, and appropriate dilutions were spread on 1.5% agar medium containing (per liter): K₂HPO₄, 11.2 g; KH₂PO₄, 4.8 g; (NH₄)₂SO₄, 2.0 g; MgSO₄·7H₂O, 0.2 g; glucose, 10 g; thiamine, 1 mg; and streptomycin, 10 mg. Recombinants were counted at 48 hr. In the experiments reported in Tables 1 to 3, there were 10⁸ to 2 × 10⁸ cells each of strains C600B and 1895 at the end of the incubation period. In the experiments in Tables 4 to 8 and Fig. 1, there were 2 × 10⁷ to 8 × 10⁷ cells of strain C600B and 10⁸ to 2 × 10⁸ cells of strain 1895.

Bacterial cell wall was prepared (Salton and Horne, 1951) by grinding 5 g (wet weight) of cells with 15 g of alumina in a mortar. The resulting paste was suspended in 25 ml of 0.01 M tris(hydroxymethyl)aminomethane (Tris; pH 7.4)-0.01 M magnesium acetate and centrifuged twice at 3,000 × *g* for 10 min; the precipitates were discarded. The supernatant fluid was centrifuged at 10,000 × *g* for 10 min, and the precipitate was washed three times with 0.1 M phosphate (pH 7.2), followed by three additional washings with water.

The inhibitor was solubilized from cell wall by incubating 100 mg (dry weight) of cell wall in a 10-ml final volume containing 1.0 mmole of phosphate (pH 7.0) and 0.1 mmole of NaIO₄. After incubation for 30 min at 37 C, the insoluble materials were removed by centrifugation at 15,000 × *g* for 20 min, and the supernatant fluid was dialyzed for 5 hr against 6 liters of water, followed by

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an overnight dialysis against 6 liters of 0.01 M phosphate at pH 7.0.

The inhibitor was solubilized from whole cells by incubating 10 g (wet weight) of cells in a final volume of 30 ml containing 3 mmoles of phosphate (pH 7.0) and 3.0 mmoles of NaIO_4 . After incubation for 30 min at 37 C, 30 μg of deoxyribonuclease were added, and the incubation was continued for an additional 5 min. The remainder of the procedure is identical to that described for the solubilization from cell wall. The soluble inhibitor was stored at -15 C.

Ribonuclease, deoxyribonuclease, trypsin, and lipase were obtained from Worthington Biochemicals Corp., Freehold, N.J.; and lipoxidase and papain, from Nutritional Biochemicals Corp., Cleveland, Ohio. Pronase and spermine were obtained from Calbiochem; protamine from Eli

Lilly & Co., Indianapolis, Ind.; and heparin, from Hynson, Westcott and Dunning, Inc., Baltimore, Md. Lysozyme from hen egg white was a generous gift of Marshall Nirenberg of the National Institutes of Health.

The following assays were used: anthrone (Ashwell, 1957); thiobarbituric acid (Warren, 1959); orcinol (Dische, 1955b); diphenylamine (Dische, 1955a); Elson-Morgan (Boas, 1953); phosphate (Ames and Dubin, 1960); protein (Lowry et al., 1951).

RESULTS

Acid and alkali treatment. The pH stability of the inhibitor was studied prior to attempts to solubilize it from cell wall. Table 1 shows that it was relatively acid labile, being almost completely inactivated in 0.01 N HCl at 80 C for 5 min, and that the cell-wall preparations derived from male and female cells were not detectably different in this regard. The relative alkali stability of the cell-wall inhibitor and the similarity in behavior of male and female cell wall was indicated by the results of experiments 1 and 2 of Table 2. Treatment of the cell wall with 1.0 N NaOH for 1 hr at 100 C resulted (experiment 3) in a marked enhancement of the inhibitory activity of both male and female cell wall. The development of inhibitory activity by *A. faecalis* cell wall, only after such treatment, emphasizes the importance of assessing the specificity of the effect of any given treatment. (It is not known whether the inhibition observed with *A. faecalis* represents

TABLE 1. Effect of acid treatment on the cell-wall inhibitor*

Addition	Recombinants per 10^{-3} ml			
	Un-treated	0.01 N HCl (5 min)		
		40 C	60 C	80 C
None.....	297	—	—	—
Cell wall C600.....	22	17	101	218
Cell wall 1895.....	33	18	86	167

* Cell wall (1 mg) was treated as shown and then neutralized and assayed as described in Materials and Methods.

TABLE 2. Effect of alkali treatment on the cell-wall inhibitor*

Expt no.	Addition	Untreated	Treated with NaOH					
			NaOH concn	Time	Temp			
					40 C	60 C	80 C	100 C
1†	None	495	0.01	5				—
	Cell wall C600	83						168
	Cell wall 1895	70						122
	<i>Alcaligenes faecalis</i> cell wall	398						385
2†	None	102	0.01	5	—	—	—	
	Cell wall C600	8			11	7	9	
	Cell wall 1895	7			7	7	9	
3	None	283	1.0	60				—
	Cell wall C600	15						1‡
	Cell wall 1895	26						3‡
	<i>A. faecalis</i> cell wall	322						1‡

* Results are expressed as recombinants per 10^{-3} ml.

† Experiments 1 and 2 were performed as described in Table 1.

‡ Cell wall (10 mg) in 0.5 ml of 1.0 N NaOH was incubated 1 hr at 100 C. The samples were cooled, neutralized, and the equivalent of 1.0 mg of cell wall was assayed as described in Materials and Methods.

TABLE 3. *Solubilization of the cell-wall inhibitor with sodium meta periodate**

Expt no.	Addition	Recombinants per 10^{-4} ml†
1	None	159 (0.0)
	Cell wall C600	27 (1.0), 44 (0.2), 113 (0.04)
	Periodate sediment	164 (1.0), 152 (0.2), 155 (0.04)
	Periodate supernatant fluid	31 (1.0), 115 (0.2), 128 (0.04)
2	None	191
	Cell wall 1895	25 (1.0), 73 (0.2), 144 (0.04)
	Periodate sediment	249 (1.0), 216 (0.2), 204 (0.04)
	Periodate supernatant fluid	41 (1.0), 118 (0.2), 145 (0.04)

* The procedure used for the treatment of the cell wall with periodate, as well as the conjugation assay conditions, are described in Materials and Methods.

† The values in brackets correspond to the dry weight (milligrams) of cell wall or its equivalent in the form of periodate sediment or supernatant fluid.

the formation or unmasking of a cell-wall component bearing structural similarity to the *E. coli* inhibitor, or is due to formation or exposure of a structurally unrelated moiety which interferes with conjugation at another site.)

Solubilization of the cell-wall inhibitor. Periodate treatment of cell wall proved to be the most satisfactory of several methods for the solubilization of the conjugation inhibitor. After periodate treatment, the $15,000 \times g$ sediment was completely devoid of inhibitor with good recovery of the activity in the supernatant fluid (Table 3). Under these conditions, about 7 to 8% of the dry weight of the cell wall was solubilized. At periodate concentrations between 5×10^{-2} and 5×10^{-4} M, there was no difference in the behavior of male and female cell wall. The specificity of the soluble product was established by the fact that *A. faecalis* did not yield inhibitor activity when treated with periodate.

Urea (8 M) and deoxycholate (0.5%) were much less efficient in solubilizing the inhibitor from cell wall; about 80% of the activity was retained in the sediment. Treatment with 90% phenol was also characterized by small yields.

Solubilization of inhibitor from whole cells. To bypass the logistic problems attendant to the preparation of cell walls, attempts were made to solubilize the inhibitor directly from whole cells. As shown in Table 4, soluble conjugation inhibitor can be derived from whole cells. Evidence for the specificity of this phenomenon was the total inactivity of such fractions derived from *M. lysodeikticus* (Table 4), as well as from *A. faecalis*, *S. aureus*, and *S. albus*. Since there was no difference in the behavior of male and female cells, the remainder of this study was performed with the soluble inhibitor obtained only from female cells.

Confidence in the assumption that the solubil-

TABLE 4. *Solubilization of the conjugation inhibitor from whole cells with periodate**

Periodate-soluble material added	Recombinants per 10^{-4} ml	
	Without inhibitor	With inhibitor
None.....	1,310	—
C600B (0.05 ml).....	—	172
C600B (0.20 ml).....	—	125
<i>Micrococcus lysodeikticus</i> (0.1 ml).....	—	1,180
<i>M. lysodeikticus</i> (0.5 ml).....	—	1,330

* The procedures for the periodate treatment of whole cells and the conjugation assay are described in Materials and Methods.

ized inhibitor and that associated with cell wall are the same was obtained from the following observations: (i) both are acid-labile and alkali-stable and (ii) the inhibitory effect of both types of preparations was maximal when added at the onset of mating, being essentially ineffectual when added 10 min after mixing male and female cells.

Figure 1 illustrates a typical titration of the soluble material. Approximately 90% inhibition was obtained with about 40 μ g of dry weight (4.0 μ liters). The steepness of the linear portion of the inhibition curve (0 to 1.0 μ liters) as well as the error inherent in bacterial plate counting assays emphasizes the need for careful titration of the inhibitor following any given procedure to determine loss of activity.

When the soluble inhibitor was subjected to centrifugation at $100,000 \times g$ for 1 hr, it was not sedimented (Table 5). However, after 3 hr most of the inhibitory activity was found in the pellet and in that portion of the supernatant fluid adjacent to the bottom of the centrifuge tube.

FIG. 1. Titration of inhibitory activity of periodate-solubilized inhibitor.

TABLE 5. Centrifugation of periodate-solubilized inhibitor to at $100,000 \times g$ *

Fraction no.	Recombinants per 10^{-3} ml	
	with inhibitor fraction centrifuged at $100,000 \times g$	
	1 hr	3 hr
	—	—
1	80	800
2	210	540
3	160	620
4	180	590
5	150	490
6	120	190
Pellet	150	160

* The periodate-solubilized inhibitor in 0.01 M phosphate (pH 7.0) was centrifuged for the time shown, and a series of 2.0-ml samples were removed from the top of the tube. The pellet was then suspended in 2.0 ml of 0.01 M phosphate (pH 7.0). Equivalent volumes of each of the fractions were tested in the conjugation assay described in Materials and Methods. Without inhibitor, the number of recombinants was 670.

Further titration of the samples taken after 3 hr at $100,000 \times g$ revealed that less than 1% of the original inhibitor remained in the topmost sample (2.0 ml), whereas the resuspended pellet was enriched approximately fivefold. It is interesting to note that the inhibitor solubilized by phenol treatment behaved in a similar manner.

The finding that the ionic strength of the mating mixture could affect the soluble inhibitor (Table 6) is in contrast to the fact that the cell wall-associated inhibitor, as well as the mating reaction itself, were unaffected in this range of ionic strength. Although 0.1 M Tris or phosphate at pH 7.0 abolished inhibitor activity, it was

possible to recover active inhibitory by dialysis against 0.01 M phosphate (pH 7.0).

Effect of enzymes and cationic reagents on the soluble inhibitor. Since the careful control of ionic strength was of importance in assessing activity of the soluble inhibitor, the treatments described below were all followed by dialysis against 0.01 M phosphate at pH 7.0. The inhibitor was not detectably affected by incubation for 1 hr at 37 C with a 100 μg/ml concentration of the following enzymes: deoxyribonuclease, ribonuclease, lipase, lipoxidase, trypsin, papain, pronase, or a crude *Clostridium perfringens* extract. [This preparation was a gift of Bertram Gesner, New York University School of Medicine, and is known (Stack and Morgan, 1949) to contain a variety of carbohydrases.] Although lysozyme inactivated the inhibitor (Table 7), it is unlikely that the

TABLE 6. Effect of ionic strength on the periodate-solubilized conjugation inhibitor*

Addition	Recombinants per 10^{-3} ml
None.....	590
Inhibitor.....	110
Inhibitor + 25 mM NaCl.....	160
Inhibitor + 50 mM NaCl.....	390
Inhibitor + 75 mM NaCl.....	420
Inhibitor + 100 mM NaCl.....	470
Inhibitor + 200 mM NaCl.....	410

* Periodate-solubilized inhibitor (0.4 mg) in 0.01 M phosphate (pH 7.0) in the presence of the indicated concentrations of NaCl was assayed by the procedure described in Materials and Methods.

TABLE 7. Effect of lysozyme on the periodate-solubilized conjugation inhibitor*

Amt of lysozyme μg/ml	Initial incubation at 37 C hr	Recombinants per 10^{-3} ml	
		Without inhibitor	With inhibitor
None	0	559	—
None	2	—	163
50	1	532	561
25	1	—	106
25	2	—	118

* The initial incubation mixture contained 45 μmoles of Tris (pH 7.0), inhibitor, and the indicated amounts of lysozyme in 1.5 ml. At the end of the incubation period, the samples were chilled and dialyzed for 18 hr against 0.01 M phosphate (pH 7.0); 0.5 ml of the dialyzed material was tested in the conjugation assay described in Materials and Methods.

enzyme was exerting its effect in a catalytic manner; incubation with 25 μ g of enzyme for 2 hr was without effect, whereas incubation with 50 μ g of enzyme for 1 hr resulted in the neutralization of the inhibitor. Lysozyme itself, at the concentrations used and in the conjugation conditions employed (Table 7), had no effect upon the recombination frequency in this and several other confirmatory experiments.

Consistent with the suggested noncatalytic function of lysozyme are the findings shown in Table 8, which demonstrate that protamine and other cationic reagents also neutralized the inhibitor. Protamine, a known lethal agent for some bacteria (Miller et al., 1942), markedly suppressed the formation of recombinants in the absence of inhibitor (Table 8, experiment 2). At similar concentrations, it was lethal for the strains used in these experiments, and the percentage loss of bacterial viability was sufficient to explain the lower rate of recombination. When protamine and the soluble inhibitor were both present in the proper proportions in a mating mixture, the lethal effect of protamine and the inhibitory effect of the conjugation inhibitor cancelled each other (Table 8, experiment 2).

Addition of lysozyme, protamine, spermine, or manganese to the soluble inhibitor resulted in the formation of a gross precipitate. In all cases in which a precipitate was produced, a concomitant loss of inhibitor activity resulted. Attempts to recover activity from the precipitates were unrewarding, e.g., when the manganese precipitate was dialyzed against ethylenediaminetetraacetate and then 0.01 M phosphate at pH 7.0, only 10% of the activity was recovered.

Since the reagents which neutralized the inhibitor are known to react with polyanions, a series of polyanionic substances were evaluated both for their ability to inhibit mating and for their capacity to neutralize inhibitor. *E. coli* soluble ribonucleic acid (RNA), yeast RNA, and heparin were tested at levels of up to 5 mg/ml and were found to be completely ineffectual. Polyglutamic acid inactivated the inhibitor only when present at 5.0 mg/ml and was without effect when tested in the mating reaction in the absence of inhibitor.

Other properties. The lyophilized soluble inhibitor was not affected by extraction at 37 C with ethanol, ethanol-ether (3:1), ether, or chloroform.

The absorption spectrum of the soluble inhibitor was essentially identical to that previously reported for *E. coli* cell wall (Salton and Horne, 1951).

Although the soluble product was absorbed to

TABLE 8. Effect of protamine, spermine, and manganese ion on the periodate-solubilized inhibitor*

Expt no.	Addition to initial incubation mixture	Recombinants per 10^{-3} ml	
		Without inhibitor	With inhibitor
1	None	620	220
	Protamine (50 μ g/ml)	810	590
	Spermine (0.006 M)	560	780
	MnCl ₂ (0.03 M)	660	630
2	None	559	106
	Protamine (100 μ g/ml)	61	696

* The initial incubation mixture contained inhibitor and 45 μ moles of Tris (pH 7.2) in 1.5 ml; the indicated concentrations of protamine, spermine, or MnCl₂ were added. At the end of a 1-hr incubation period at 37 C, the samples were chilled and dialyzed for 18 hr against 0.01 M phosphate (pH 7.0); 0.5 ml of the dialyzed materials was tested in the conjugation assay described in Materials and Methods.

Norit A at pH 2.0, and less efficiently at pH 7.0, it was not possible to elute the activity with ethanol in various concentrations from 5 to 95%, with 50% ethanol-0.1 M NH₄OH, with 5 to 50% ethanol-0.1 M borate (pH 7.0 or 10.0), or with 10 to 50% ethanol-0.1 M phosphate (pH 7.0 or 8.0).

Assays performed on the soluble inhibitor produced the following results (micromoles per milligram of material) with the reference compounds shown in parenthesis used as standards: anthrone, 0.42 (glucose); Elson-Morgan, 0.16 (glucosamine); organic phosphate, 0.37 (orthophosphate); thiobarbituric acid, 0.01 (*N*-acetylneuraminic acid); diphenylamine, 0.14 (deoxyribose); orcinol, 0.27 (ribose); protein, 0.005 (bovine serum albumin). (The absorption maximum after diphenylamine treatment is at 662 m μ , which should be compared with the value obtained with deoxyribose where the maximum is 595 m μ .) When the inhibitor was partially absorbed at pH 7.0 on Norit A, there was no selective loss in any of the above assays relative to the loss of inhibitory activity.

DISCUSSION

Sneath and Lederberg (1961) reported that the conjugal substance of viable male and female cells exhibits a differential lability to periodate treatment and that males treated in this manner could behave as feeble females. It should be noted that their assay procedure did not distinguish

between the solubilization and destruction of the male "conjugal substance." Therefore, it is possible that our inability to distinguish male from female by periodate treatment may have been due to the presence, in preparations from males, of both male and female conjugation inhibitor. Experiments in which soluble male and female inhibitor were mixed failed to demonstrate any neutralization of inhibitory activity. However, the possible presence of male and female inhibitor in the male preparations makes interpretation of these results difficult.

The fact that the variety of enzymes tested did not neutralize the inhibitor suggests that it is not solely protein or nucleic acid. The inability to extract the activity with standard organic solvents, as well as the absence of any effect with lipase and lipoxidase, tends to exclude many lipids as being the sole source of inhibitor activity. However, it should be noted that our data do not exclude the possibility that a periodate-insensitive polysaccharide alone could be responsible for the inhibitor activity. Clearly, interpretation of the data pertaining to the effects of cationic reagents, ionic strength, and acid and alkali treatment cannot be made without more precise information concerning the overall structure of the soluble inhibitor.

Although the sequence of events between the initial effective association of male and female cells and the transfer of deoxyribonucleic acid is poorly understood, it is tempting to speculate that the soluble conjugation inhibitor and the sexual receptor site are one and the same.

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