Role of Outer-membrane Proteins and Lipopolysaccharide in Conjugation between *Neisseria gonorrhoeae* and *Neisseria cinerea*

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Little is known concerning the mechanism involved in cell contact between the donor and recipient during conjugation in *Neisseria gonorrhoeae*. The formation of stable mating pairs during conjugation in *Escherichia coli* appears to require a specific protein as well as LPS in the outer membrane of the recipient cell. To attempt to identify the cell surface components necessary for conjugation in the neisseriae, we began a comparison of the outer membrane of *Neisseria cinerea* strains that can (Con⁺) and cannot (Con⁻) serve as recipients in conjugation with *N. gonorrhoeae*. There were no differences in outer-membrane protein profiles on SDS-polyacrylamide gel electrophoresis between Con⁺ and Con⁻ strains that could be correlated with the ability to conjugate. However, whole outer membrane isolated from Con⁺ strains specifically inhibited conjugation while those from Con⁻ strains did not. Proteolytic cleavage of outer-membrane proteins by trypsin, pronase or α -chymotrypsin abolished the inhibitory effect of Con⁺ outer membranes, suggesting that these outer membranes contained a protease-sensitive protein(s) involved in conjugation. Although periodate oxidation of Con⁺ outer-membrane carbohydrates did not abolish the inhibitory action of these membranes, purified LPS from both Con⁺ and Con⁻ strains inhibited conjugation when added at low concentrations. These results suggest that conjugation requires the presence of a specific conjugal receptor that consists of both LPS and one or more outer-membrane proteins. Both Con⁺ and Con⁻ strains contain the necessary LPS, but only Con⁺ strains contain the required protein(s).

INTRODUCTION

Transfer of DNA from one bacterial cell to another by conjugation requires physical contact between donor and recipient cells (deHann & Gross, 1962). In *Escherichia coli*, conjugative plasmids specify sex pili (filamentous appendages composed of protein subunits: Achtman & Skurray, 1977) that are required for the establishment of early mating contact between donor and recipient cells (Curtiss, 1969). The donor and recipient then form stable mating pairs or aggregates, through extensive wall-to-wall contacts.

The role of specific surface components on the recipient cell during conjugation by a variety of bacterial species has been widely studied. *E. coli* F⁻ mutants that are deficient in conjugation (Con⁻) (Achtman *et al.*, 1978; Havekes & Hoekstra, 1976; Henning *et al.*, 1976; Schweizer & Henning, 1977) cannot form stable mating aggregates in liquid, but can form mating aggregates stable enough to allow DNA transfer on solid surfaces. Henning *et al.* (1976) have shown that the gene product of the *ompA* cistron is needed for cells to function efficiently as recipients with F-carrying donor cells. The *ompA* gene product, OmpA protein, is believed to be necessary for stabilization of mating aggregates rather than acting as a receptor for the F pilus. LPS is also

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thought to be involved in the ability of a cell to serve as a recipient in conjugation. LPS may play a role in I-specific conjugation equivalent to that of OmpA in F-specific conjugation (Havekes *et al.*, 1977a, 1978).

Conjugation in *Neisseria gonorrhoeae* is different from that of *E. coli* F, in that a plasmid-encoded pilus has not been identified, and transfer only occurs when cells are on a solid surface (Roberts & Falkow, 1977). The gonococcal β -lactamase plasmid can be transferred by conjugation from *N. gonorrhoeae* to some, but not all, strains of *Neisseria cinerea* (Genco *et al.*, 1984). Thus the host-range of the gonococcal conjugal plasmid is quite limited. Virtually nothing is known about the surface structures required in the donor or recipient in the gonococcal conjugation system. An understanding of the basis for the limited host range is of particular interest now that the TetM marker, which has been shown to be on a conjugal transposon in other genera (Franke & Clewell, 1981), has been found inserted into the gonococcal conjugal plasmid (Morse *et al.*, 1986).

We have used transformation to introduce a derivative of the β -lactamase plasmid into an *N. cinerea* strain that cannot accept the plasmid by conjugation, ruling out a barrier at the level of plasmid replication (Genco & Clark, 1988). Here we describe studies designed to define the role of outer-membrane proteins and LPS in the ability of different *N. cinerea* strains to serve as recipients with *N. gonorrhoeae* as donor during conjugation.

METHODS

Strains and growth conditions. The bacterial strains used in this study are listed in Table 1. *N. gonorrhoeae*, *N. cinerea* and *E. coli* strains were grown as described in the preceding paper (Genco & Clark, 1988).

Isolation of outer membranes. Outer membranes of *N. cinerea* strains were prepared from overnight cultures grown on GCK agar, as previously described for *N. gonorrhoeae* (Clark *et al.*, 1987). Outer-membrane pellets were suspended in water, and protein content was determined by the Lowry method with bovine serum albumin as a standard.

Inhibition of conjugation. *N. gonorrhoeae* CDC67 donor cells (10^7) were suspended in GCK broth + 100 μ g DNAase ml⁻¹ and incubated for 2 h at 37 °C in 5% (v/v) CO₂-in-air on a polycarbonate membrane (Nucleopore) with or without the addition of either sarkosyl-insoluble outer membranes or purified LPS species. *N. cinerea* recipient cells (10^8 , rifampicin resistant) were then added and incubation was continued for an additional 2 h. Transconjugants were selected on GCK + 0.25 μ g penicillin and 1.0 μ g rifampicin ml⁻¹. Transconjugants were confirmed by detection of β -lactamase activity with the chromogenic cephalosporin nitrocefin (Glaxo Research) (O'Callaghan *et al.*, 1972).

Treatment of outer membranes with proteolytic enzymes or periodate. Proteolytic cleavage of sarkosyl-insoluble membranes was achieved using 50 or 100 μ g trypsin (Sigma), 25 or 50 μ g pronase (Calbiochem-Behring), and 25 or 50 μ g α -chymotrypsin (Sigma), as described by Blake *et al.* (1981). Each enzyme was incubated with a 100 μ g

Table 1. *Bacterial strains used in this study*

Organism	Strain	Relevant phenotype*	Source†
<i>N. gonorrhoeae</i>	CDC67	Tra ⁺ Bla ⁺ Rif ^s	CDC, Atlanta
<i>N. cinerea</i>	RUN4029	Bla ⁻ Con ⁻ Rif ^r	NRL 30003
<i>N. cinerea</i>	RUN4030	Bla ⁻ Con ⁺ Rif ^r	NRL 32165
<i>N. cinerea</i>	RUN4031	Bla ⁻ Con ⁺ Rif ^r	NRL 32824
<i>N. cinerea</i>	RUN4032	Bla ⁻ Con ⁻ Rif ^r	NRL 33295
<i>N. cinerea</i>	RUN4033	Bla ⁻ Con ⁺ Rif ^r	NRL 32828
<i>E. coli</i>	HB101	Bla ⁻ Con ⁺	H. Boyer

* Tra⁺ strains contain the gonococcal conjugal plasmid, pLE2451, and Bla⁺ strains contain the 4.4 MDa β -lactamase plasmid, pFT1. Con⁻ and Con⁺ indicate the inability or capability, respectively, of the strain to acquire the β -lactamase plasmid by conjugation with *N. gonorrhoeae*. Rif^s and Rif^r indicate that the strain is sensitive or resistant, respectively, to 1 μ g rifampin ml⁻¹.

† NRL denotes Neisseria Reference Laboratory.

protein sample of freshly prepared outer membrane for 1 h at 37 °C. Samples were diluted 1:10 with 0.25 M-Tris/HCl, pH 7.2, and centrifuged at 100 000 *g* for 2 h. The resulting pellet of each preparation was suspended in 100 µl distilled water and used immediately or divided into portions and stored at -70 °C.

Periodate oxidation of sarkosyl-insoluble membranes (100 µg protein) was achieved by the addition of 20 nmol sodium metaperiodate (Mallinkrodt) in 0.05 M-sodium acetate buffer, pH 5.4 (Stein & Heath, 1979). Reactions were incubated in the dark at 4 °C for 20 h and stopped by the addition of 10 nmol glycerol.

Isolation of LPS. LPS was isolated from *N. cinerea* strains by a modification of the hot phenol/water extraction procedure of Westphal & Jann (1965) as modified by Johnston *et al.* (1976). The LPS pellet was dissolved in 1.0 ml distilled water, lyophilized to a dry powder and stored at 4 °C. LPS (rough type) was isolated from *E. coli* HB101 as previously described (Palermo *et al.*, 1987) and *Salmonella minnesota* LPS (smooth type) was purchased from Sigma.

SDS-polyacrylamide electrophoresis (SDS-PAGE). This was performed on 13% (w/v) gels with 4% stacking gels as described by Laemmli (1970). LPS preparations were analysed by SDS-PAGE as described by Schneider *et al.* (1984).

RESULTS

Effects of trypsin treatment on conjugal transfer

We were interested in identifying the surface structures necessary for a cell to serve as a recipient in conjugation with *N. gonorrhoeae*. Preincubation of *N. cinerea* recipient cells with trypsin (250 or 500 µg per 10⁹ cells in 1 ml), followed by incubation with soybean trypsin inhibitor prior to conjugation with an *N. gonorrhoeae* donor, resulted in a decrease in the frequency of conjugal transfer of the gonococcal β -lactamase plasmid (data not shown). The addition of trypsin to recipient cells decreased the number of viable cells by 45%; however, the conjugation frequency was decreased by 95% and thus these results are not due simply to a decrease in the viability of recipients. Although the effects of trypsin on recipient cells may be pleiotropic, one of the major effects is the proteolytic cleavage of exposed outer-membrane proteins.

N. cinerea outer-membrane proteins

The decrease in the transfer of the gonococcal β -lactamase to *N. cinerea* recipient cells pretreated with trypsin suggested that an intact outer-membrane protein(s) of a Con⁺ strain played a role in its ability to serve as a recipient with *N. gonorrhoeae*. Therefore, we began a comparison of the outer membranes from Con⁺ and Con⁻ *N. cinerea* strains by examining the outer-membrane protein composition by SDS-PAGE (Fig. 1). All five *N. cinerea* strains produced different outer-membrane protein patterns on SDS-PAGE. However, no simple correlation existed between the presence of a particular outer-membrane protein and the ability to act as a recipient in conjugation with *N. gonorrhoeae*. Silver staining of the gel also did not reveal any protein whose presence could be correlated with the Con⁺ phenotype (data not shown).

Inhibition of conjugation of N. cinerea

When *N. gonorrhoeae* CDC67 was preincubated with outer-membrane preparations from *N. cinerea* RUN4030, 4033 and 4031 (Con⁺ strains), and then added to *N. cinerea* RUN4030 recipient cells, inhibition of conjugation was observed (Fig. 2). While low concentrations of outer-membrane preparations from *N. cinerea* RUN4030, 4031 and 4033 strongly inhibited conjugation, much higher concentrations of outer-membrane preparations from Con⁻ strains (RUN4029 and RUN4032) were required for inhibitory effects. Complete inhibition of conjugation was observed at 2.0 µg of RUN4030, at 3.0 µg of RUN4031 and RUN4033, and at 5.0 µg of RUN4029 and RUN4032 outer-membrane protein (data not shown). One microgram of outer-membrane protein is equivalent to approximately 10⁷ cells, which is the number of viable donor cells used in the conjugation inhibition experiments.

Fig. 1. SDS-PAGE of sarkosyl-insoluble outer membranes of *N. cinerea*. Electrophoresis was performed on 13% gels with 4% stacking gels (Laemmli, 1970) and the gel was stained with Coomassie blue. Strains: A, RUN4030 (Con⁺); B, RUN4031 (Con⁺); C, RUN4033 (Con⁺); D, RUN4029 (Con⁻); E, RUN4032 (Con⁻).

Since trypsin treatment of whole recipient cells inhibited conjugation, outer-membrane preparations from *N. cinerea* Con⁺ organisms were treated with various proteolytic enzymes in an attempt to identify proteins important for conjugation. Outer-membrane preparations were also treated with sodium metaperiodate to degrade LPS. Periodate treatment of outer membranes from *N. cinerea* RUN4030 slightly increased the inhibitory action of these preparations when 0.75 µg outer-membrane protein was added, but had no effect at 1.5 µg (Table 2). Treatment of these outer-membrane preparations with each of the three proteolytic enzymes completely abolished the inhibitory action of these preparations on conjugation at both 0.75 and 1.5 µg added outer-membrane protein. Trypsin treatment of outer membranes from RUN4031 and RUN4033 also decreased the inhibitory action of these preparations on conjugation, though not as completely as seen with outer membranes from RUN4030. Trypsin treatment of the Con⁻ strains, RUN4029 and RUN4032, decreased the inhibitory action of these outer-membrane

Fig. 2. Inhibition of conjugation by outer-membrane preparations. *N. gonorrhoeae* CDC67 donor cells were incubated for 2 h with outer-membrane preparations from the five different *N. cinerea* strains. *N. cinerea* RUN4030 recipient cells were added and conjugation mixtures were incubated for an additional 2 h. The conjugation inhibition index was determined by dividing the conjugation frequency for donors preincubated with outer-membrane preparations by the conjugation frequency for donors preincubated without the addition of outer membranes. The values are from one experiment and are representative of three separate experiments. Outer membrane preparations were from: ▲, RUN4032; ●, RUN4029; △, RUN4033; ◐, RUN4031; ○, RUN4030.

Table 2. Effects of treatments of *N. cinerea* outer membranes on their ability to inhibit conjugation between *N. gonorrhoeae* CDC67 donors and *N. cinerea* RUN4030 recipients

Source of outer membranes	Treatment*	Conjugation inhibition index†	
		0.75 µg	1.5 µg
RUN4030	None	0.39	0.19
	Periodate	0.30	0.19
	Trypsin 50 µg	1.0	0.80
	Trypsin 100 µg	1.1	1.0
	Pronase 25 µg	1.2	0.85
	Pronase 50 µg	1.2	1.0
	α-Chymotrypsin 25 µg	1.0	1.2
	α-Chymotrypsin 50 µg	1.2	1.1
RUN4031	None	0.36	ND
	Periodate	0.19	ND
	Trypsin 100 µg	0.89	ND
RUN4033	None	0.40	ND
	Periodate	0.38	ND
	Trypsin 100 µg	0.60	ND

ND, Not determined.

* Treatments were carried out as described in Methods.

† Conjugation inhibition index was calculated as described in the legend to Fig. 2, and the values represent the mean of two independent experiments. Values of 0.75 µg or 1.5 µg indicate the amount of outer-membrane protein that was used in the conjugation experiments, after treatment of a 100 µg sample with the indicated agent.

Fig. 3. SDS-PAGE of *N. cinerea* LPS preparations. Purified LPS samples from *N. cinerea* [0.5 µg (a) and 1.0 µg (b)] were brought to 25 µl with distilled water, 25 µl of 2 × LPS-SDS-PAGE sample buffer was added, and the samples were boiled for 5 min and subjected to SDS-PAGE on 13% separating gels (without added SDS) (Schneider *et al.*, 1984). Gels were stained with an LPS-specific silver stain (Tsai & Frasch, 1982). Strains: A, RUN4030 (Con⁺); B, RUN4031 (Con⁺); C, RUN4033 (Con⁺); D, RUN4029 (Con⁻); E, RUN4032 (Con⁻). Lanes on the far left and right are smooth-type LPS from *S. minnesota*.

preparations that was observed only at much higher concentrations (data not shown). Periodate oxidation of RUN4031 and RUN4033 outer membranes slightly increased the inhibitory effect of these outer membranes on conjugation.

Inhibition of conjugation by purified LPS

To define more closely the role of LPS in the ability of *N. cinerea* to serve as recipients in conjugation, LPS from each of the *N. cinerea* strains was purified and analysed by SDS-PAGE (Fig. 3). All five *N. cinerea* strains appeared to produce a rough-type LPS. No simple correlation could be made between the LPS banding pattern from those strains which could and could not serve as recipients in conjugation with *N. gonorrhoeae*. Two of the Con⁺ strains, RUN4030 and RUN4031, did not produce a higher molecular mass band; however, the LPS species from Con⁺ RUN4033 did contain this higher molecular mass band as did Con⁻ RUN4029 and RUN4032.

Table 3. *Inhibition of conjugation by LPS*

Strain from which LPS isolated	LPS (μ g)*	Conjugation inhibition index†
<i>N. cinerea</i> RUN4030	1.0	0.50
<i>N. cinerea</i> RUN4031	1.0	0.44
<i>N. cinerea</i> RUN4033	1.0	0.64
<i>N. cinerea</i> RUN4029	1.0	0.40
<i>N. cinerea</i> RUN4032	1.0	0.36
<i>E. coli</i> HB101	1.0	1.1
	10	1.1
<i>S. minnesota</i>	1.0	1.2
	10	1.0

* With 0.5 μ g LPS from each *N. cinerea* strain, no inhibition of conjugation was observed (index = 1.0).

† Conjugation inhibition index was calculated as described in the legend to Fig. 2, and values represent the mean of two independent experiments.

These LPS species were examined for their possible role in conjugation by preincubating them with donors. These LPS/donor mixtures were then added to *N. cinerea* RUN4030 recipient cells. At 0.5 μ g added LPS from all *N. cinerea* strains, no inhibition of conjugation was observed. However, at 1.0 μ g LPS from both Con⁺ and Con⁻ strains, there was significant inhibition of the transfer of the β -lactamase plasmid by conjugation to *N. cinerea* recipients (Table 3). The addition of 5 μ g LPS from Con⁺ or Con⁻ strains resulted in an increased, but not complete, inhibition of conjugation (data not shown). No apparent difference in the inhibition exerted by LPS from Con⁺ versus Con⁻ strains was evident. The addition of rough *E. coli* or smooth *S. minnesota* LPS at either 1.0 or 10 μ g did not inhibit the transfer of the β -lactamase plasmid by conjugation to *N. cinerea* recipients, suggesting that the inhibition was specific for neisserial LPS.

DISCUSSION

The ability of outer-membrane preparations from Con⁺ strains to specifically inhibit the transfer of the β -lactamase plasmid by conjugation suggests that Con⁺ *N. cinerea* strains possess a component in the outer membrane that may be required for the formation of mating pairs, and that this component is absent or non-functional in Con⁻ strains. Cleavage of outer-membrane preparations with proteolytic enzymes abolished the inhibition, suggesting that the component is a protein(s). Trypsin treatment of outer membranes from all five *N. cinerea* strains resulted in the cleavage of at least three and as many as five proteins, as evidenced by protein profiles on SDS-PAGE (data not shown). However, no protein was found only in the Con⁺ strains that was cleaved by trypsin. The barrier to conjugation in Con⁻ strains may be due to the absence of a specific trypsin-sensitive outer-membrane protein whose molecular mass may vary in the different Con⁺ strains examined. Alternatively, the Con⁻ strains may contain the protein but not in a functional state.

The results from the inhibition of conjugation experiments where LPS species were used are interesting in that they imply a possible role for LPS in the ability of an *N. cinerea* recipient to interact with an *N. gonorrhoeae* donor cell during conjugation. All five *N. cinerea* strains produced LPS species which, when preincubated with donor cells, resulted in an inhibition of the transfer of the gonococcal β -lactamase plasmid. Relatively low concentrations of LPS were needed for inhibition of conjugation, similar to the *E. coli* I system where LPS is believed to play an integral role in ability of a recipient cell to form mating pairs (Havekes *et al.*, 1977a, 1978). The absence of inhibition of conjugation by either *E. coli* or *S. minnesota* LPS suggests that the inhibition seen by *N. cinerea* LPS is specific. That *E. coli* HB101 LPS was not inhibitory to conjugation was somewhat surprising, since this strain can serve as a recipient in conjugation with *N. gonorrhoeae*.

Studies in the *E. coli* F system clearly demonstrate that the decrease in, or absence of, recipient ability can result from several different defects in the recipient cell. Havekes & Hoekstra (1976) found that three out of four *ompA* mutants, selected for the loss of conjugation, retained OmpA protein in the membrane. Achtman *et al.* (1978) mapped 11 Con⁻ mutants in or near the *ompA* cistron and found that many of these did not synthesize detectable levels of OmpA. The remaining mutants produced normal levels of OmpA and the authors suggested that OmpA may have been non-functional in these mutants. The Con⁻ mutants described by Achtman *et al.* (1978) were capable of binding F pili but incapable of forming stable mating aggregates as measured by a Coulter counter technique. The authors concluded that OmpA protein does not represent the receptor for F pilus binding and that conjugation between F-carrying donor cells and F⁻ recipient cells proceeds by a series of specific interactions before transfer can occur. One of these intermediate stages, that of stabilization, requires OmpA in an LPS matrix in the recipient.

In other studies (Manoil & Rosenbusch, 1982) 62 mutants were selected for their inability to act as efficient F factor recipients in conjugation. Of these, 60 were found to be altered in the amount or function of OmpA protein, while the remaining two had altered LPS. The *ompA* mutants were of three types: (1) those that lacked OmpA protein, (2) those that had greatly reduced levels of OmpA protein, and (3) those that had normal amounts of OmpA protein. The OmpA protein from the third type of mutants migrated differently from the normal protein in SDS-urea-PAGE.

Recently Ried & Henning (1987) have characterized a set of *ompA* mutants which exhibit a conjugation-deficient phenotype but produce wild-type amounts of OmpA protein. Conjugation deficiency in these mutants appears to be caused by a single amino acid substitution in the OmpA protein, glycine-154 for aspartic acid. These authors conclude that the OmpA protein is the primary recognition site for the donor during conjugation and that only a small area of the OmpA protein is recognized.

The role of LPS in the recipient cell is not as well defined as that of OmpA. However, mutants with defects in the polysaccharide core of the LPS are poor recipients for R64drd11 and other I-like plasmids (Havekes *et al.*, 1977b; Manning & Reeves, 1977). Several F⁻ mutants have been described which have altered LPS, although the decrease in transfer may be due to changes in outer-membrane proteins and/or the interaction between these proteins and LPS (Achtman *et al.*, 1978; Henning *et al.*, 1976; Puspurs *et al.*, 1983).

Duke & Guiney (1983) studied the role of LPS structure in the recipient cell using a series of well-defined LPS mutations in *S. minnesota*. They found a decrease in transfer which was correlated with the extent of the defect in LPS structure; they believe that the progressive decrease in transfer suggests that no single component of the LPS structure acts as a specific receptor and that perhaps some general property of LPS is important in conjugation. The addition of large amounts of purified LPS to mating mixtures does not block RK2-mediated transfer and argues against a role for LPS as a receptor in the recipient for mating with RK2. However, addition of purified OmpA and LPS, but not OmpA alone, greatly decreased the number of observed F-plasmid transconjugants in *E. coli* K12 (Alphen *et al.*, 1977; Schweizer & Henning, 1977).

The differences between Con⁻ and Con⁺ *N. cinerea* may reflect differences between the interaction of LPS and a specific protein(s). If a specific protein in the recipient is required for stable mating aggregate formation, the proper configuration may be essential to its activity. One of the characteristics of *E. coli* Con⁻ mutants is their ability to mate on solid surfaces. The *N. cinerea* Con⁻ strains described in our studies are not able to mate on a solid surface and, therefore, a direct comparison between *E. coli* Con⁻ mutants may not be possible. However, the results obtained with *E. coli* Con⁻ mutants may help in the correlation of possible defects in these *N. cinerea* Con⁻ strains. It is clear that most of the *E. coli* Con⁻ mutants are defective in stable mating pair formation as a result of changes in outer-membrane proteins and/or LPS structure. In our studies, results from the inhibition of conjugation experiments demonstrate a role for outer-membrane proteins and LPS in the ability of *N. cinerea* recipient cells to mate with *N. gonorrhoeae* donor cells.

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