

Export and Intercellular Transfer of DNA via Membrane Blebs of *Neisseria gonorrhoeae*

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Naturally elaborated membrane bleb material is frequently observed in cultures of *Neisseria gonorrhoeae*. This material was purified and analyzed for protein, lipopolysaccharide, and nucleic acid content. The electrophoretic protein profiles of two bleb-rich fractions, called BI and BII, were distinct, with only BII containing lipopolysaccharide and outer membrane proteins I and III. Both fractions contained RNA, circular DNA, and linear DNA. Exogenous pancreatic DNase I appeared to hydrolyze all bleb-associated DNA in fraction BI and the linear DNA in fraction BII. The circular DNA molecules associated with fraction BII resisted digestion. Electron microscopy of the bleb fractions verified their DNA content. Fixing blebs with glutaraldehyde before mounting them for microscopy prevented release of internal DNA. Such fixation produced little change in the micrographs of BI; however, only traces of DNA were observed in fixed BII preparations. Incubation of wild-type gonococci in mixtures of DNase and blebs purified from antibiotic-resistant strains resulted in efficient exchange of penicillinase-specifying R plasmids. Recipients incorporated plasmids independently of endogenous and exogenous chromosomal streptomycin resistance markers. These *in vitro* results suggest that bleb formation by *N. gonorrhoeae* may serve to transfer plasmids intercellularly *in vivo*, perhaps constituting a previously unexplored genetic exchange mechanism in these bacteria.

Like several other gram-negative pathogens, *Neisseria gonorrhoeae* forms and releases membrane vesicles, termed blebs, during growth *in vitro* (5, 8) and *in vivo* (5). Gonococcal blebs are known to contain immunodominant cell surface antigens, including protein I (PI), PII, PIII, the H.8 antigen, and lipopolysaccharide (LPS) (8). Preliminary characterizations showed that significant quantities of naturally elaborated membrane blebs are recoverable from broth culture supernatants of *N. gonorrhoeae*. Although this finding suggests active formation of these vesicles by gonococci (8), the physiological role of the vesicles is obscure.

Purified blebs equilibrate into two fractions, BI and BII, with respective densities of 1.12 and 1.30, by sucrose density gradient centrifugation (8). Because constituents unique to BI or BII might account for the density difference, we first considered whether nucleic acids were associated with bleb fractions. After finding plasmids, linear DNA, and RNA associated with blebs, we examined whether the DNA found in blebs could be incorporated and expressed by recipient gonococci.

The results showed that plasmids are sequestered within outer membrane-derived BII vesicles and resist exhaustive DNase digestion. In addition, wild-type recipient gonococci incorporated and expressed penicillinase-specifying plasmids associated with blebs when incubated in suspensions of blebs and DNase.

MATERIALS AND METHODS

Bacteria. The strains of *N. gonorrhoeae* used are listed in Table 1. Each strain was maintained by single-colony transfers on gonococcal clear typing medium (GCTM) (19) or on GCTM containing penicillin G (1.0 µg/ml; Eli Lilly & Co., Indianapolis, Ind.), streptomycin (200 µg/ml; Eli Lilly), or both, as appropriate. Transparent (O⁻) and nonpiliated (P⁻) variants were selected by colony morphology as previously

described (11, 19). Liquid cultures were grown in GCTM lacking agar (gonococcal broth) for 7 h to mid-log phase or 9 h to late-log phase.

Harvests. Sixteen-hour agar cultures of the strains were harvested and suspended in Dulbecco phosphate-buffered saline, pH 7.2 (dPBS), by vortexing. Cells were removed by centrifugation for 10 min at 10,000 × *g* in a TMS-4 rotor (Tomy, Tokyo, Japan) at 4°C. The resulting supernatant was transferred to a clean tube and centrifuged for an additional 20 min under the same conditions. This second supernatant, containing blebs, was retained.

Bleb fractions BI and BII were separated by sucrose density gradient centrifugation as follows. Step gradients of 65, 60, 50, 40, 30, 25, and 20% sucrose in dPBS were prepared in polycarbonate centrifuge tubes (7 by 34 mm; Beckman Instruments, Inc., Palo Alto, Calif.). After being layered on the gradients, the bleb suspensions were centrifuged for 1 h at 201,000 × *g* in a TLS-55 rotor (Beckman) at 4°C. Banded membranes were removed, diluted 1:1 in dPBS, and recovered by centrifugation for 30 min at 435,000 × *g* in a TLA-100 rotor (Beckman) at 4°C. Pellets were gently rinsed with and then suspended in dPBS or buffer containing 10 mM Tris and 1 mM EDTA, pH 8.0, as needed. Broth

TABLE 1. *N. gonorrhoeae* strains used

Strain	Length of plasmid harbored (kbp)	Genotype or phenotype	Source
JS1	4.2	Wild type	J. Swanson
31426	4.2, 7.1, 36	<i>bla</i> ⁺	American Type Culture Collection, Rockville, Md.
FA589	4.2, 7.1, 36	<i>bla</i> ⁺ Str ^r	P. F. Sparling
Dst11	4.2	Str ^r	FA589 chromosomes × JS1

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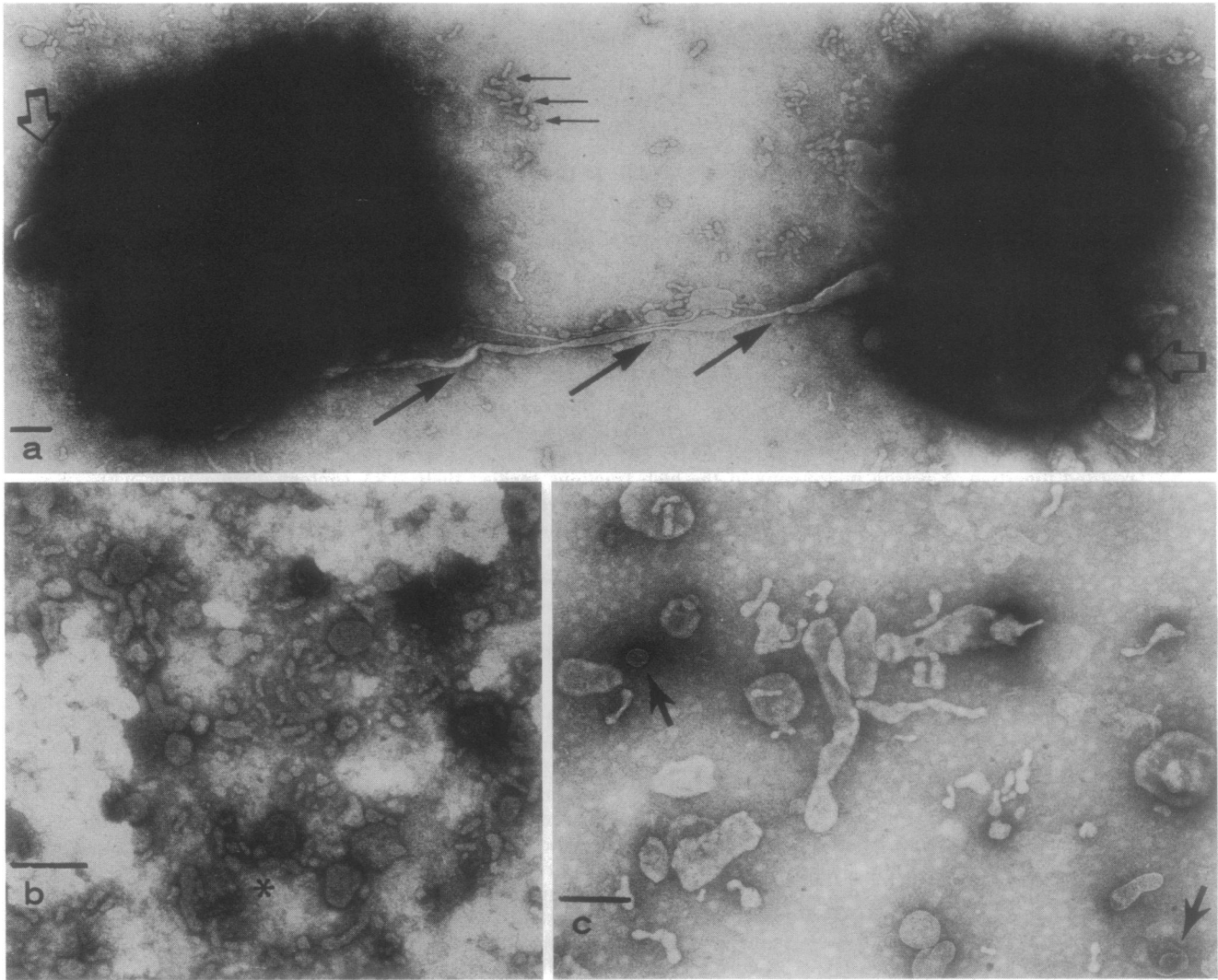


FIG. 1. Cells and blebs of *N. gonorrhoeae*. Gonococci from agar cultures and blebs, purified by differential and sucrose density gradient centrifugation, were adsorbed on coated grids, negatively stained with phosphotungstic acid, and observed by electron microscopy. Whole cells (a) retained numerous pleomorphic blebs, including spherical (\square), lobed (\leftarrow), and tubular (\blacktriangleright) vesicles. Tubular blebs appeared to interconnect some cells. Purified bleb fractions BI (b) and BII (c) contained no cells and were morphologically distinct. Membranes in BI were generally smaller and less granular than BII vesicles. Aggregates of tiny vesicles (*) sedimented with BI. Tubular blebs and blebs with densely staining cores (arrows) were concentrated in BII. Bars, 200 nm.

cultures were harvested by centrifugation for 20 min at 4,000 $\times g$.

Electron microscopy. Parlodion (Mallinckrodt, Inc., St. Louis, Mo.)-coated grids were prepared as previously described (9). Sixteen-hour colonies of *N. gonorrhoeae* were blotted onto grids, negatively stained for 10 s in 0.3% phosphotungstic acid, pH 6.5, and dried in air. Purified blebs were adsorbed on coated grids for 10 min at room temperature, washed briefly in water, and stained for 30 s as described above. The grids were observed at 75 kV on an HU-11E-1 transmission electron microscope (Hitachi, Ltd., Tokyo, Japan).

The Kleinschmidt technique for DNA microscopy, with adaptations described by Garon (9), was also used to observe dPBS suspensions of BI and BII. Both blebs cross-linked for 1 h with 2% glutaraldehyde in dPBS and untreated blebs were spread and then retrieved on coated grids. The grids were examined with a JEM-100 B transmission elec-

tron microscope (Japan Electronic Optics Laboratory, Co., Ltd., Tokyo, Japan) at 40 kV.

DNA extraction. Cellular and bleb DNAs were purified from sodium dodecyl sulfate (SDS)-proteinase K lysates by phenol-chloroform extractions as described by Maniatis et al. (16). For some experiments, RNA present in the extracts was hydrolyzed with DNase-free RNase (Sigma Chemical Co., St. Louis, Mo.) (16). Supercoiled plasmids were purified from whole-cell and bleb extracts by Nucleic Acid Chromatography System (NACS) (Bethesda Research Laboratories, Inc., Gaithersburg, Md.) chromatography as previously described (10).

DNase digestion. Suspensions of BI, BII, and pBR322 in dPBS containing approximately 2 μg of DNA per ml were incubated with pancreatic DNase I (1.0 $\mu\text{g}/\text{ml}$; 2.5 U/ μg ; Worthington Biochemicals, Freehold, N.J.) for 10 min at 25°C. Digestion mixtures were then extracted with phenol and chloroform as described above.

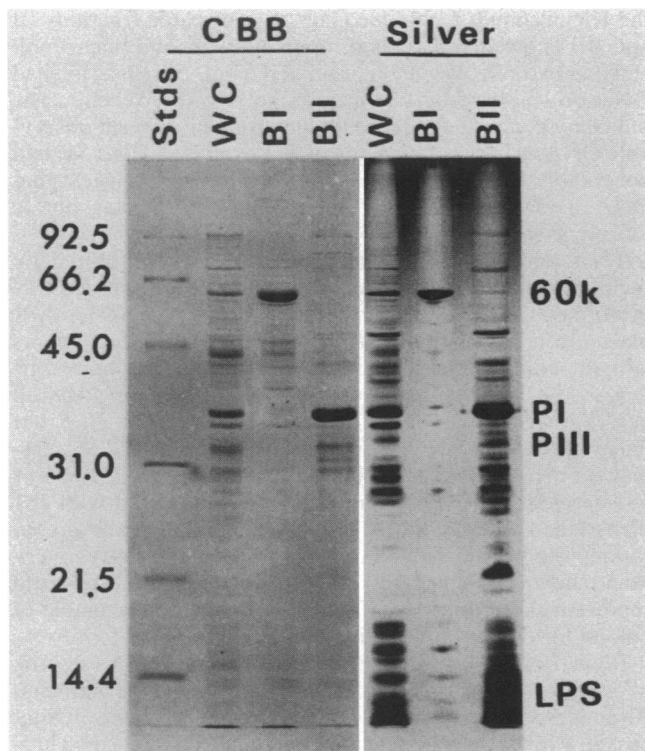


FIG. 2. Separation of whole-cell (WC) and bleb fraction BI and BII lysates in 12.5% SDS-polyacrylamide gels. Samples were stained with Coomassie brilliant blue (CBB) or silver. Profiles of BI and BII were distinct. A 60-kDa protein was highly concentrated in BI, whereas BII contained PI, PIII, and LPS. Molecular masses of standards (Stds) are given in kilodaltons.

Gel electrophoresis. Bleb proteins and LPS were compared by SDS-polyacrylamide gel electrophoresis, using the discontinuous buffer system of Laemmli (15). Specific procedures have been described elsewhere (12). Gels were stained with Coomassie brilliant blue R (Sigma) or silver. Electrophoresis of extracted DNA was performed in agarose (Bio-Rad Laboratories, Richmond, Calif.) slabs containing 0.5 μ g of ethidium bromide per ml, using Tris acetate buffer, pH 8.0 (16). Agarose gels were typically electrophoresed for 3.5 h at 40 V and 25°C.

Plasmid transfer. Donor blebs were prepared by filtering broth culture supernatants through nitrocellulose membranes with 0.22- μ m porosity (Millipore Corp., Bedford, Mass.). Pancreatic DNase I was added at a final concentration of 1.0 μ g/ml to hydrolyze free DNA. For some experiments, a 1- μ g/ml concentration of chromosomal DNA containing streptomycin resistance markers (*Str*^r) was also added. Late-log recipient cells were then suspended in this mixture to an optical density at 600 nm of approximately 0.4 and incubated for 3 to 12 h at 37°C in 5% CO₂ with gentle agitation. Equal portions were periodically removed, serially diluted in GC broth, and spread on control and selective media for counts of CFU. Only O⁻ P⁻ variants were used in these experiments.

Detection of marker transfer. Transfer of antibiotic resistance to sensitive recipients was inferred by sustained growth on selective media. Transfer efficiency was determined by comparing CFU on control and selective plates. Several recipient clones from each experiment were maintained for verification of R-plasmid incorporation by electro-

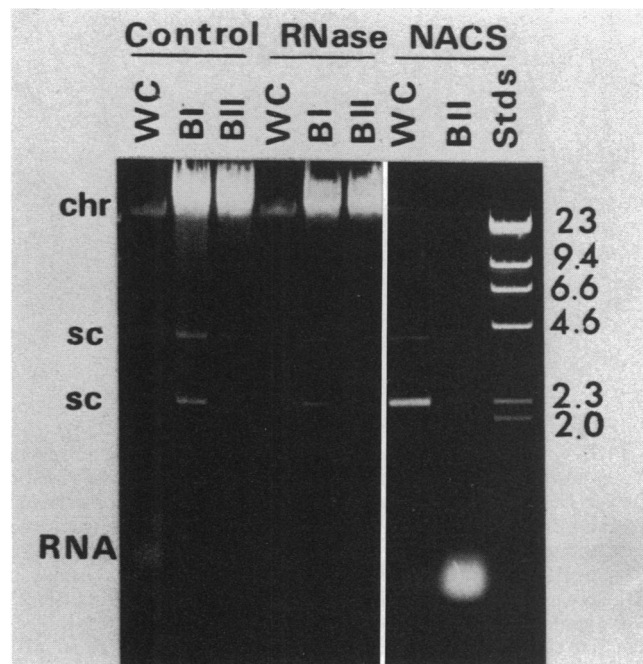


FIG. 3. Agarose gel electrophoretic separations of whole-cell (WC) and bleb nucleic acids. Nucleic acid extracts of *N. gonorrhoeae* 31426 cells and bleb fractions BI and BII were separated and stained with ethidium bromide. Shown is a comparison of crude extracts with extracts hydrolyzed with DNase-free RNase and NACS-purified supercoiled DNA molecules. The presence of RNA in each extract was indicated by hydrolysis with RNase A. Chromosomal DNA (chr) and supercoiled (sc) and nicked plasmids were recovered from cells and blebs. Plasmids resolved corresponded in size to pFA1, pFA2, and pFA3 found in *N. gonorrhoeae*. Molecular size standards (Stds), indicated in kilobase pairs, were *Hind*III fragments of bacteriophage lambda.

phoresis and penicillinase production by Beta-test strips (Medical Wire and Equipment Co., New York, N.Y.). Experimental controls included similar colony counts of *bla*⁺ cells incubated with donor or recipient blebs, wild-type recipients incubated with recipient cell blebs, and *bla*⁺ donor blebs spread directly on GCTM.

RESULTS

Spherical, lobed, and tubular blebs were observed attached to and surrounding gonococci grown on solid medium (Fig. 1a). Tubular membranes appeared to form interconnections between membranes and cells, often separated by more than one cell diameter. Each morphological class of vesicle exhibited wide variation in dimensions.

Gonococcal bleb fractions BI and BII were purified from culture harvests by differential centrifugation, followed by sucrose density gradient centrifugation. Fractions BI (Fig. 1b) and BII (Fig. 1c) were rich in membranes and devoid of cells. Most BI vesicles appeared flattened and lobed and exhibited little surface texture. Aggregates of vesicles measuring 12 to 14 nm in diameter were also observed in BI. The dimensions of 25 randomly selected BI blebs measured from 12 to 66 by 24 to 152 nm (average, 26 by 62 nm).

Fraction BII vesicles (Fig. 1c) were morphologically distinct from BI vesicles. Typical BII blebs appeared spherical to elongated or tubular. Granular staining patterns suggested that the membranes were textured or contained pits. Spherical BII membranes ranged from 6 to 206 nm in diameter

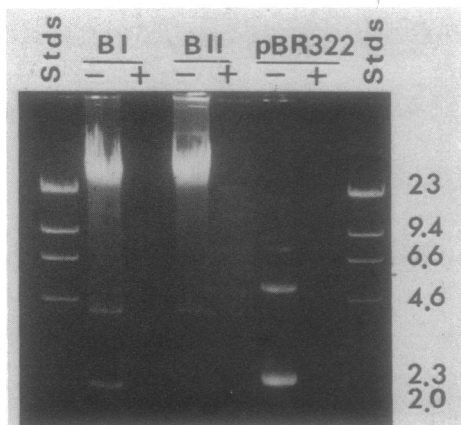


FIG. 4. Effects of DNase on bleb-associated DNA. Blebs from *N. gonorrhoeae* 31426 were incubated for 10 min in pancreatic DNase I (1 $\mu\text{g}/\text{ml}$) before separation in sucrose density gradients. Extracts of DNA from each hydrolyzed fraction (+) were compared with extracts of undigested controls (-). All DNA associated with BI and BII chromosomal DNA was nuclease sensitive. Plasmids in BII were protected from hydrolysis. Equivalent digestion of pBR322 (2.0 $\mu\text{g}/\text{ml}$) resulted in complete loss of detectable plasmids. Molecular size standards (Stds), indicated in kilobase pairs, were *Hind*III fragments of phage lambda.

(average, 78 nm). Tubular blebs ranging from 18 to 60 by 72 to 500 nm (mean, 30 by 196 nm) were also observed.

Proteins associated with gonococcal cells and blebs were compared by SDS-polyacrylamide gel electrophoresis (Fig. 2). The three preparations were clearly distinct. Whole-cell lysates contained LPS and the major proteins typical of $\text{O}^- \text{P}^-$ variants (11, 19), including 60- and 44-kilodalton (kDa) proteins and outer membrane proteins PI and PIII. The 60-kDa band and PI were noticeably segregated into BI and BII, respectively. Reduction-sensitive PIII and LPS were concentrated in BII, and immunoblots detected the H.8 antigen in this fraction (data not shown). The relatively few proteins seen in both fractions included bands at 14, 30, 34, and 44 kDa.

Electrophoretic analysis of nucleic acids purified from cells and blebs of strain 31426 showed that plasmids, heterogenous chromosomal DNA, and RNA were present in each extract (Fig. 3). Each preparation contained bands corresponding in size to gonococcal plasmids pFA1, pFA2, and pFA3, at 4.2, 36, and 7.1 kilobase pairs (kbp) (3, 4, 6, 7, 18), respectively. Supercoiled forms of each plasmid were resolved after purification by NACS chromatography. The presence of RNA in cells and blebs was indicated by sensitivity to DNase-free RNase. A higher ratio of DNA to RNA was evident in blebs than in cells.

Two sets of experiments were done to determine whether bleb DNA was enclosed within the vesicles or simply copurified with them. These two possibilities were resolved by comparing the susceptibilities of bleb DNA and pBR322 to hydrolysis by an excess of pancreatic DNase I. Figure 4 is an agarose gel containing extracts of BI, BII, and pBR322, showing the effects of a 10-min digestion with 1.0 μg of DNase I per ml on the DNA in each sample. Whereas complete hydrolysis of pBR322, BI DNA, and chromosomal DNA in BII occurred, BII plasmids appeared unaffected by the enzyme.

The location of bleb-associated DNA was also determined by direct electron microscopic examination of blebs, using

the Kleinschmidt technique (Fig. 5). Untreated fractions BI and BII showed supercoiled, open circular, and linear molecules. Partially lysed blebs in BII (Fig. 5b, inserts) had DNA protruding from the membrane vesicles. When cross-linked by glutaraldehyde treatment, BI (Fig. 5c) had observable DNA, whereas few fields of BII examined (Fig. 5d) had observable DNA. These results taken together suggest that DNA is packaged within BII membrane vesicles but is loosely associated with BI membranes.

To determine whether bleb DNA was incorporated by recipient gonococci, a series of bleb-mediated crosses was performed (Table 2). The experiments showed transfer of R plasmids in blebs of *bla*⁺ strains to wild-type JS1 recipients without concomitant transformation by chromosomal *Str*^r DNA. JS1 cells incubated with strain 31426 blebs acquired penicillin resistance at an average rate of 6.5×10^{-3} but failed to incorporate *Str*^r markers. Donor 31426 cells remained penicillin resistant during incubation with 31426- or JS1-derived blebs. Recipient strain JS1 incubated with JS1 blebs failed to grow on penicillin G, and 31426 bleb preparations showed no growth on GCTM. The marker recovery rates shown are averages of three independent trials and represent the proportion of viable recipient cells capable of colony formation on selective media.

Blebs from strain FA589 (*bla*⁺ *Str*^r) were used as donors to assay possible transfer of chromosomal determinants. Although crosses between FA589 and JS1 resulted in successful R-plasmid transfer, recipient clones expressing chromosomal *Str*^r markers were recovered only after incubation with exogenous *Str*^r DNA in the absence of DNase. Recipients incubated with blebs from strain Dst11, a streptomycin-resistant transformant of JS1, also failed to express *Str*^r determinants.

DISCUSSION

This study shows that plasmid and chromosomal DNAs from *N. gonorrhoeae* associate with elaborated membrane vesicles termed blebs. Plasmids associated with bleb fraction BII were resistant to exhaustive enzymatic hydrolysis and were likely enclosed within the vesicles. Furthermore, wild-type gonococci incubated with DNase, as well as blebs carrying penicillinase-specifying plasmids, incorporated and expressed these markers. These data suggest that bleb formation represents an effective, previously unexplored mechanism of plasmid export and exchange between gonococci.

Previous work showed that gonococcal blebs segregate into fractions BI and BII during sucrose density gradient centrifugation (8). The results presented here confirm those findings and provide a more detailed characterization of the fractions by morphology, protein and nucleic acid profiles, and nuclease resistance. Fractions BI and BII consisted of a morphologically diverse collection of membrane vesicles that were observable on and around bacteria grown on solid media. Although BI vesicles were generally smaller than BII vesicles, pleomorphism in each fraction made microscopic analysis of fraction purity difficult. Pleomorphism was extensive in fraction BII, which contained membrane vesicles of wide-ranging dimensions. Membrane texture, however, correlated with membrane density. Relatively dense BII membranes exhibited granular surfaces, whereas BI vesicles appeared smooth.

Further differences were identified by SDS-polyacrylamide gel electrophoresis profiles. BI and BII showed distinct differences in the concentrations of LPS and major proteins, including a 60-kDa protein, PI, and PIII. The

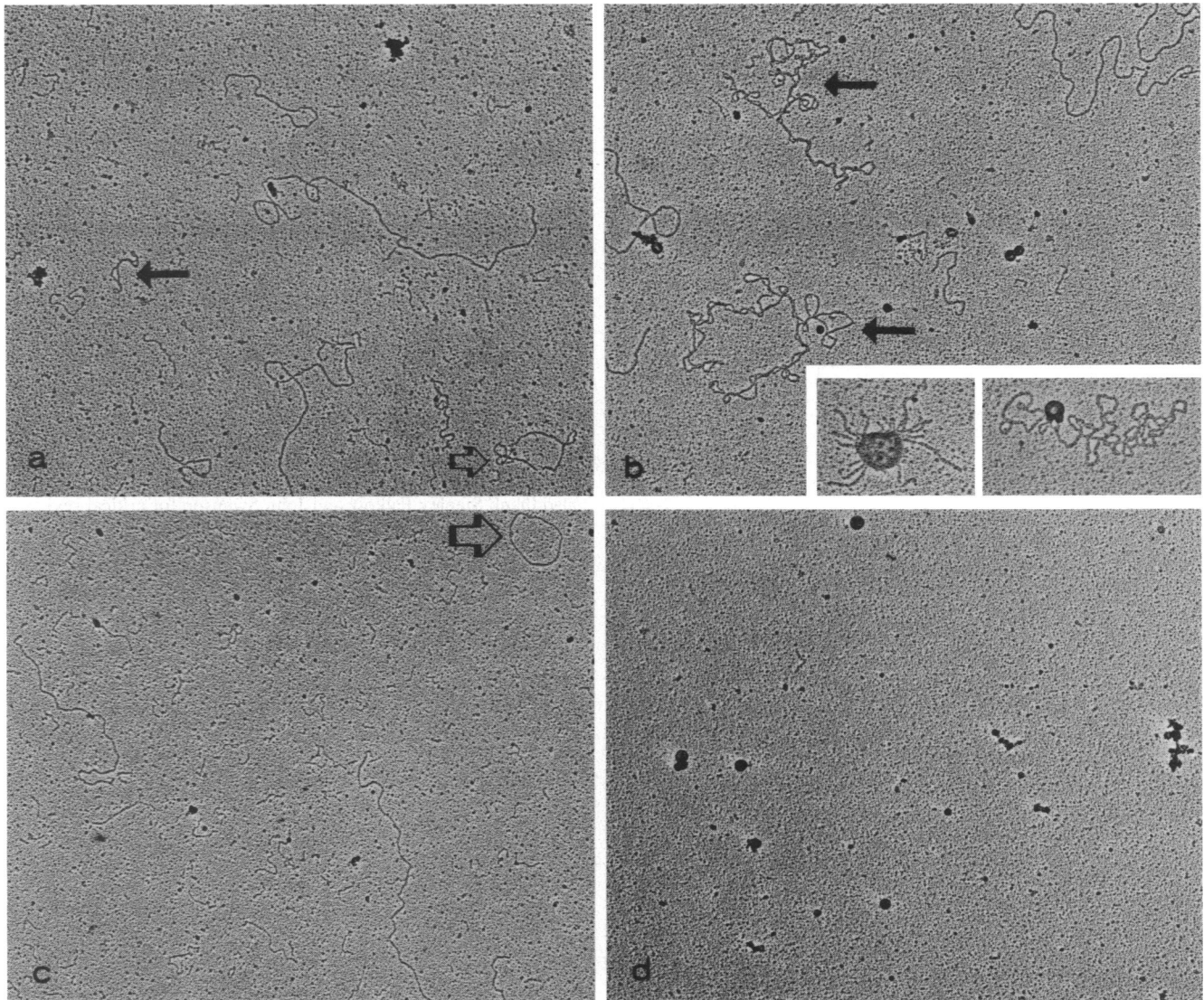


FIG. 5. Effects of fixation on Kleinschmidt microscopy of bleb-associated DNA. Control and glutaraldehyde-cross-linked blebs from BI and BII were prepared by the aqueous Kleinschmidt technique for DNA electron microscopy. Linear DNA and open-circle (\rightarrow) and supercoiled (\leftarrow) plasmids were present in BI (a), BII (b), and fixed BI (c) preparations. Partially lysed BII vesicles had protruding DNA (b, inserts). Only traces of DNA were observed in fixed BII samples (d).

profile of BII, containing LPS, PI, and PIII, is consistent with published profiles of gonococcal outer membranes (11, 19), and the identities of PI and PIII were confirmed by reactivity with an anti-PIb monoclonal antibody and reduction sensitivity, respectively (data not shown). The lack of those proteins and clear concentration of the 60-kDa protein in BI suggests that BI membranes originate elsewhere, perhaps as plasma membrane fragments. Such segregation of proteins in the fractions conflicts with earlier observations of similarity in the protein profiles of BI and BII (8). The study described here separated blebs washed from surfaces of agar-cultured cells, whereas the previous study separated blebs that were recovered from broth culture supernatants by ultracentrifugation. It is possible that incomplete resuspension of high-speed pellets before sucrose density separation may have caused cross-contamination of the fractions in the earlier study.

Both BI and BII contained chromosomal DNA and all plasmids carried by the gonococci producing the blebs. In

strain 31426, these included a 4.2-kbp cryptic plasmid (3, 6, 14), a 36-kbp conjugative plasmid (6, 18), and a 7.1-kbp penicillinase-specifying R plasmid (4, 6, 7, 17). Blebs also contained relatively small amounts of RNA. Such results suggest that packaging of nucleic acids in blebs may be regulated rather than random.

A major aim of this study was to determine whether DNA is packaged within membrane blebs or is only randomly associated with blebs, perhaps by cell lysis. Previous work has shown that mid-log-phase bleb pellets contain few if any ribosomal proteins or antigens, which would likely accumulate in such pellets if cell lysis were occurring (D. W. Dorward, Ph.D. dissertation, University of Montana, Missoula, 1987). The experiments described here showed that plasmids in BII were protected from hydrolysis by sufficient DNase I to degrade both 2 μ g of pBR322 per ml and all linear, open-circle, and supercoiled DNAs associated with BI. Electron microscopy revealed DNA protruding from partially lysed BII membranes. In addition, plasmid and

TABLE 2. Bleb-mediated transfer of plasmid without chromosomal markers

Bleb donor	Incubation condition ^a			Marker recovery rate	
	Recipient cell	Str ^r DNA	DNase	<i>bla</i> ⁺	Str ^r
	JS1	+	-	<10 ⁻⁸	1.4 × 10 ⁻⁵
	JS1	+	+	ND ^b	<10 ⁻⁸
JS1	JS1	-	+	<10 ⁻⁸	<10 ⁻⁸
JS1	31426	-	+	1.3	ND
31426	31426	-	+	2.7	<10 ⁻⁸
31426		-	-	NG ^c	NG
31426	JS1	+	-	3.3 × 10 ⁻³	7.6 × 10 ⁻⁶
31426	JS1	+	+	1.4 × 10 ⁻³	<10 ⁻⁸
FA589	JS1	+	-	4.0 × 10 ⁻³	1.7 × 10 ⁻⁵
FA589	JS1	+	+	1.1 × 10 ⁻²	<10 ⁻⁸
FA589	JS1	-	+	7.3 × 10 ⁻³	<10 ⁻⁸
Dst11	JS1	-	+	ND	<10 ⁻⁸

^a DNA and DNase concentrations were 1 µg/ml.

^b ND, Not determined.

^c NG, No growth.

linear DNAs were observed in micrographs of lysed BII preparations, whereas very little DNA was observable in preparations of cross-linked membranes that failed to lyse. Similar preparations of BI showed little change by cross-linking. These results suggest that the DNA is packaged within BII membranes but is loosely associated with BI. The nature of the association of DNA and BI is unknown, but BII DNA appears to be exported by cells.

Extensive review of the literature revealed no other reports of cellular DNA exported within bacterial membrane vesicles. Gonococcal blebs do, however, exhibit similarities to *Haemophilus* transformasomes (1, 2, 13). Both originate as membrane evaginations from the cell surface (1, 2, 8, 13), and the DNA contained in both resists exogenous nucleases (1, 2, 13). Transformasomes apparently contribute to *Haemophilus* transformation by sequestering transforming DNA before becoming internalized to release the enclosed DNA within the cytoplasm (1, 2, 13). There are some distinctions between blebs and transformasomes. Reportedly, only mutant strains of *Haemophilus influenzae* release transformasomes (2), but all gonococcal strains thus far examined release blebs (5, 6, 8). In addition, DNA detected in transformasomes originated exogenously (1, 2, 13). The extent of homology between these systems is being studied, with particular interest in determining whether blebs were involved in the plasmid exchange between *Haemophilus* and *Neisseria* spp. suggested by several studies (6, 7, 17).

Results of bleb-mediated plasmid transfer experiments suggested that export of bleb-associated DNA may serve a genetic exchange role for *N. gonorrhoeae*. Wild-type JS1 cells readily incorporated and expressed the *bla* marker of R plasmids contained in cell-free bleb suspensions. Conjugative plasmids, absent in JS1, were also exhibited by all recipient clones analyzed, though it is uncertain whether conjugative and R plasmids are transferred concurrently or separately in multiple-transfer events. The study did not address the possible transfer of the 4.2-kbp cryptic plasmid.

Plasmid transfer occurred despite the addition of DNase I to incubation mixtures. Control experiments that found no Str^r clones resulting from transfers involving either Str^r bleb donors or incubations with DNase and transforming *str* DNA are consistent with results showing that blebs protect plasmids but not chromosomal DNA. Such results also indicated that the exogenous DNase was sufficient to prevent transformation by naked DNA.

These experiments were performed with O⁻ P⁻ variants only. Variants with O⁺ and P⁺ phenotypes autoagglutinate (11, 19), making CFU determinations inaccurate. The possible effects of these variations on bleb formation and bleb-mediated genetic exchange are unknown.

These initial in vitro experiments suggest that bleb-mediated plasmid transfer may contribute to plasmid exchange among gonococci in vivo. Further experiments on the regulation of DNA export in blebs and the compatibility of various strains, species, and genera for bleb-mediated genetic exchange should provide the basis for a model describing this process. Preliminary studies suggest that intercellular transfer of plasmids, via blebs, can occur between *Haemophilus* and *Neisseria* spp. and that this process may be widespread among gram-negative bacteria.

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