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Transformation-mediated exchange of virulence determinants by co-cultivation of pathogenic Neisseriae

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1. SUMMARY

The horizontal flow of genetic material between microbes utilizes three principal routes: conjugation, transduction and transformation. While the significance in nature of the first two pathways is generally accepted, the in vivo role of transformation remains uncertain, despite the early observations by Griffith in 1928 on the transformation of streptococci from an avirulent to a virulent state [1]. Recently, circumstantial evidence was collected suggesting a role for transformation-mediated horizontal exchange in the modulation of virulence determinants of pathogenic Neisseriae and the variation of surface structures. In order to further assess the significance of transformation-mediated exchange we performed simple co-cultivation experiments of different *Neisseria* strains. We observed an efficient intra- and interspecies transfer of essential virulence determinants; the process was sensitive to the presence of DNaseI in the culture and was blocked in transformation-deficient recipients.

2. INTRODUCTION

The pathogenic Neisseriae, i.e. *N. gonorrhoeae* and *N. meningitidis*, representing Gram-negative diplococci, infect humans as their sole natural host. Several non-pathogenic, commensal *Neisseria* species also exist which still carry some of the putative neisserial virulence determinants, such as *opa*, *pil*, and *lip* [2]. Like many other bacterial species, the pathogenic Neisseriae are naturally competent for genetic transformation [3,4]. This process is very efficient, involving a number of bacterial functions including the major pilus subunit, PilE [5] and an intact *dud1* locus [6]. In order to allow efficient DNA uptake, the bacteria must recognize a specific sequence motif in the transforming DNA [7], which often represents part of the structure of transcriptional terminators, as in the case of the *opa*, *iga* and *ctr* genes [8–11]. The uptake of DNA into the bacterial cytoplasm occurs via linear intermediates [6]. Subsequent incorporation into the chromosome

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or re-circularization of plasmids therefore requires RecA-dependent homologous recombination. Both the uptake specificity and the recombination requirement imply that only species-related DNA can be stably accomodated in a recipient cell. The various restriction modification systems existing in Neisseriae [12] may impose additional constraints on the transformation process.

Recent in vitro experiments suggest a role for natural transformation in the phase and antigenic variation of the *N. gonorrhoeae* pilin [5,13] although pilin variation can also occur by an alternative intracellular pathway [5,14] (for review see ref. 15). Moreover, the observation of mosaic genes, such as *iga* and *penA* [9,16], provides strong evidence for an ongoing process of horizontal exchange among various *Neisseria* species. The association of DNA uptake sequences with such virulence determinants suggests that horizontal exchange may be driven by transformation. Conversely, transducing phage or conjugative systems that would allow the mobilization of chromosomal genes have not been reported for Neisseriae. We therefore set out in this investigation to further elucidate the potential significance of transformation as a mediator of genetic exchange in vivo. Due to the restricted host specificity of the pathogenic and most commensal *Neisseria* species, and their inability to multiply and survive outside their host, this addresses the question of whether transformation is likely to occur during the course of an infection.

In order to assess the transformation-mediated exchange between two *Neisseria* strains, we chose different chromosomal determinants, all of which have been implicated in pathogenesis: the *iga* determinant, a single copy gene present in both gonococci and meningococci, codes for an extracellular enzyme that specifically cleaves human IgA1 antibody [17,18]. The *opa* determinants constitute a family of polymorphic genes which are present in about 11, three to four, and up to two copies in gonococci, meningococci, and certain commensal isolates, respectively [2,9,10]; these genes encode phase variable outer membrane proteins that play essential roles in the interaction with human epithelial cells or polymorphonuclear leukocytes ([19,21]; E.M. Kupsch, B. Knepper, T. Kuroki, I. Hener and T.F. Meyer, submitted). A third virulence determinant used in these studies are the capsule-synthesis genes of *N. meningitidis* (*cps*) which are absent in gonococci but present in several commensal species [22]. They are essential for the production of a capsule which is composed of acidic polysaccharides. The capsular types of *N. meningitidis* differ in their chemical composition and their immunological characteristics, a single strain, however, has only a single capsule-gene locus [22].

3. RESULTS AND DISCUSSION

In a first set of experiments, two *N. gonorrhoeae* MS11 strains were co-cultivated in order to assess the genetic transfer of the *opaE* locus [23]. The donor N246 carried an insertion of the *ermC* determinant derived from *Bacillus subtilis* [24] in the *Cla*I site of the *N. gonorrhoeae* MS11 *opaE* gene [9], and carried, in addition, a *recA* locus partially replaced by the cat_{GC} cassette to abolish its own transformation competence (D. Facius and C.P. Gibbs, unpublished) [25]. The transformation-competent recipient N248 was an S-pilin variant of strain MS11 [5] and carried two chromosomal markers, nal^r and rif^r. Donor and recipient strains were first grown separately and then combined at an OD_{550} of about 0.2. From the combined culture, aliquots were taken at serveral time points in order to determine the number of Erm^r transformants resulting from the transfer of the *opaE::ermC* determinant from the donor to the recipient. As shown in Fig. 1, efficient marker transfer occurred as early as 15 min after combining the cultures, reaching a maximum of 10^{-5} Erm^r transformants per recipient cells after 2 h of co-cultivation. No Erm^r transformants were obtained by co-cultivation of donor and recipient in the presence of DNaseI, indicating that the observed marker exchange resulted from transformation. Genomic DNA of individual transformants was prepared to confirm by Southern hybridization that the *ermC* marker was inserted in one of the recipient's *opa* loci (data not shown). This provided evidence for recombinant *opa* loci that were generated simply by co-cultivation of the two strains.

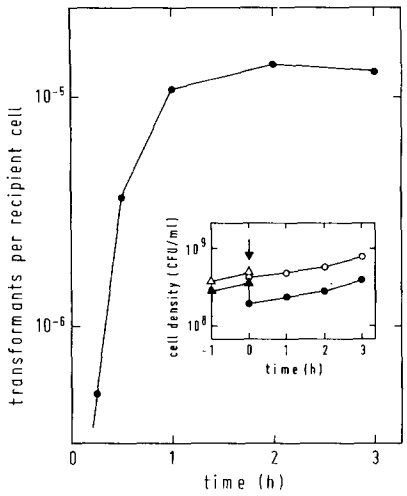

Fig. 1. Kinetics of Erm^r-resistance-marker transfer between two co-cultivated *N. gonorrhoeae* strains. Figure insert: growth curves of the MS11-N246 donor strain (*opaE::ermC*, *recA::cat*$_{GC}$; open symbols) and the MS11-N248 recipient (S[= S-pilin], *nal*^{r}, *rif*^{r}; filled symbols), cultured in proteose peptone transformation medium containing 10 mM $MgCl_2$ [5,25]. At zero time, the two separate cultures (triangles) were combined (circles); the number of donor and recipient cells in the combined culture was determined on the basis of resistances expressed. Main figure: ratio between Erm^r (7 μg/ml), Nal^r (6 μg/ml), Rif^r (100 μg/ml) transformants and recipient cells. Erm^r resistant transformants were selected for on plates containing DNaseI (30 μg/ml) in order to abrogate transformation after plating. In the presence of 40 μg/ml DNaseI in the culture the number of transformants was below 10^{-8}.

Equivalent results were obtained using donor strain N247, identical with N246 except that it carried the *ermC* insertion in the *Bgl*II site of the IgA protease locus (*iga*) [9] (D. Facius, unpublished). With this strain the overall efficiency of the marker transfer was three-fold lower as compared with the *opaE::ermC* donor N246, a slight difference which may in part reflect the fact that *iga* represents a single copy gene in contrast to *opa*.

Next we asked for the possibility of interspecies exchanges of related genes by assaying for the DNase-sensitive transfer of the *opaE::ermC* determinant from gonococci to the non-capsulated group B meningococcal recipient N215 (*cps*$^-$, *nal*^{r}, *rif*^{r}) [26]. In these experiments we measured transformation frequencies in the order of 10^{-6}, suggesting that *opa* genes can be readily exchanged via transformation also between the two pathogenic species. In addition, evidence for the occurrence of this process was obtained for combinations of pathogenic and commensal *Neisseria* species (data not shown).

Another series of experiments was concerned with the question of whether the capsular polysaccharide phenotype of *N. meningitidis* can be altered by transformation. The *cps* gene complex is organized in distinct regions coding general functions including regulation, subunit polymerisation and transport. The biosynthesis determinant for the serotype-specific building blocks is spatially separated within the *cps* complex and differs from the equivalent region of other serotype isolates [22]. Conceivably, exchange of the serotype-specific fragment (region A) should lead to serotype conversion and this might possibly occur by transformation. To test this hypothesis, we performed a co-cultivation experiment using a serogroup W135 *N. meningitidis* strain as donor and a serogroup B strain as recipient. The latter carried chromosomal *nal*^{r} and *rif*^{r} markers while the donor was transformation-incompetent and carried an insertion of *ermC* in region D. Thus, the insertion was located adjacent to the region of interest, i.e. the serotype-specific region A, different to above described experiments involving *opa* and *iga*. The insertion did not affect the capsular polysaccharide phenotype expression. Upon co-cultivation for a period of 2 h, according to the above described protocol, Erm^r, Rif^r and Nal^r colonies grew up at a frequency of 5×10^{-6}. About one third of the transformants, which originally belonged to serogroup B, now reacted with a monoclonal antibody specific for the serogroup W135 meningococcal capsular polysaccharide. The remaining Erm^r transformants still produced the authentic serogroup B capsule indicating that these cells had only acquired the *erm*^{r} marker linked with region D and not the adjacent serogroup-specific region A of the W135 donor. Since no colonies with the triple resistance were obtained by cultivation in the presence of DNaseI the process was based on transformation.

The serogroup-converted transformants were investigated by Southern-blot analysis, using the cloned *cps* gene cluster of a serogroup B strain as

probe (Fig. 2). All transformants showed restriction patterns significantly different from both the serogroup W135 donor and the serogroup B recipient: in clone W1, for example, two *Hin*dIII fragments with sizes of about 9.5 kb each and a 3.8-kb fragment representing the serogroup-specific region A, characteristically found in the *cps* locus of the serogroup B meningococcal strain [22], were replaced by 20-kb, 8-kb and 2-kb *Hin*dIII fragments of the serogroup W135 strain. In contrast, transformant W2 exhibited only minor changes in the *cps* locus that were still sufficient to alter the capsular polysaccharide phenotype, i.e. the region A-specific 3.8-kb *Hin*dIII fragment and a 9.5-kb *Hin*dIII fragment

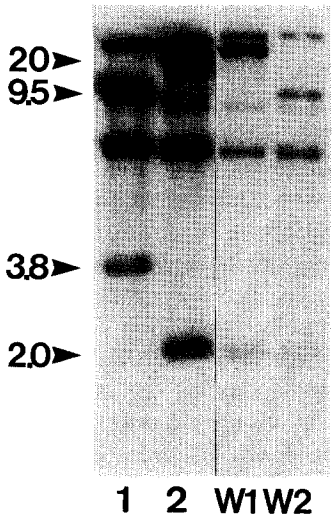

Fig. 2. Southern-blot hybridization of *N. meningitidis* chromosomal DNA using the cloned *cps* gene cluster as probe. DNA samples were cleaved using *Hin*dIII: lane 1, serogroup B-specific recipient strain derived from *N. meningitidis* B1940, U. Berger, Heidelberg; lane 2, serogroup W135-specific donor strain derived from *N. meningitidis* W135, American Type Culture Collection; lanes W1 and W2, two Erm^r transformants. Hybridization was performed with digoxigenin-labelled plasmid pMF32.35, which harbours the complete *cps* locus of *N. meningitidis* B1940 [22]. Size markers in kilobase pairs (kb) are indicated on the left. The 3.8-kb fragment of the strain recipient (lane 1) corresponds to the biosynthesis region A and one of the 9.5-kb fragments corresponds to region D and the major part of region B [22]. Thus, loss of the 3.8-kb *Hin*dIII fragments in the Erm^r transformants (W1 and W2), as compared with the recipient (lane 1), reflects changes in the capsular polysaccharide phenotype of the transformants.

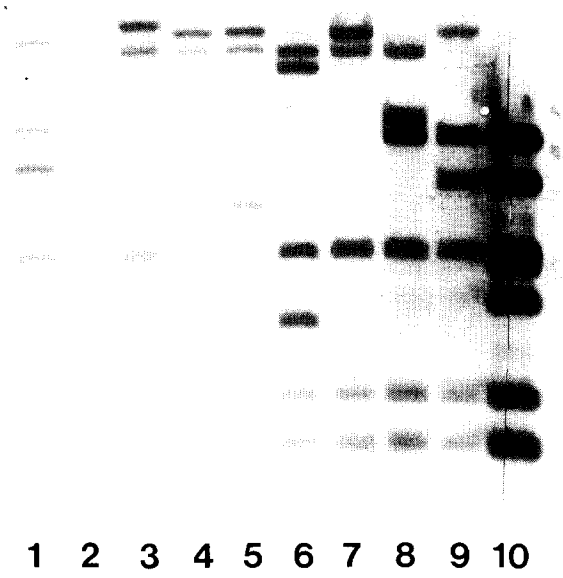

Fig. 3. Southern-blot hybridization of *Eco*RI-digested chromosomal DNA of natural *N. meningitidis* serogroup B isolates. Strains were isolated in the diagnostic laboratories of the Institut für Medizinische Mikrobiologie, Hannover (lanes 1–9). Lane 10 represents the cloned plasmid pMF32.35 [22]. Digoxigenin-labelled plasmid pMF32.35 was used as probe.

were replaced by the 2-kb and 8-kb fragments of the W135 strain (Fig. 2).

Among the transformants several different restriction patterns were observed. This phenomenon appears plausible in the context of restriction-site polymorphisms in the *cps* complexes of different serogroup isolates [11]. In the described experiment it also reflects the fact that different transformants have acquired different portions of the *cps* gene cluster from the donor strain. If transformation-driven exchanges of *cps* genes play a role in vivo, similar polymorphisms in the *cps* gene cluster of naturally isolated group B meningococci should be seen. That this is in fact the case is shown in Fig. 3, which indicates a fundamental difference to *Escherichia coli* where even different serotypes exhibit very similar restriction patterns [27]. These observations, and furthermore the occurrence of meningococcal isolates possessing different capsular types within a single clonal type [28], provide strong evidence

for an ongoing horizontal exchange process in vivo, involving parts of the *cps* gene cluster.

Our data demonstrate a remarkable efficiency and versatility of transformation-mediated processes in a *Neisseria* culture. Transformation hence may play a significant role in nature and cause the reassortment of virulence determinants among members of the genus *Neisseria* [9,16,20,31]. In fact, no other known genetic exchange mechanism exists in Neisseriae that could account for the observed in vivo phenomena.

Transformation-mediated exchange may allow pathogenic organisms to adapt to their host, but may also incidentally enhance their pathogenic potential. For example, only some capsular types have strong pathogenic potential and are preferentially associated with meningitis [29]. Up to 30% of the human population carry meningococci in their nasopharyngeal tract without being diseased. This typical situation then reflects a proper balance between the infectious agent and its host. By occasional horizontal exchange and gene reassortment, in individuals being superinfected by a second *Neisseria* strain, new situations may arise that mark the beginning of local outbreaks and epidemics [30].

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