JOURNAL OF BACTERIOLOGY, June 1979, p. 762-769
0021-9193/79/06-0762/08$02.00/0

Vol. 138, No. 3

Genetic Basis for Colonial Variation in *Neisseria gonorrhoeae*

LENA NORLANDER,* JOHN DAVIES, ANDERS NORQVIST, AND STAFFAN NORMARK

Department of Microbiology, University of Umeå, S-901 87 Umeå, Sweden

Received for publication 26 January 1979

When the piliated colony types of *Neisseria gonorrhoeae*, which predominate in recent isolates, were nonselectively subcultured in vitro, they gave rise to large numbers of nonpiliated, avirulent colonial variants. Evidence is presented to show that most of this variation occurs after active growth has ceased and that the variation is sensitive to the action of deoxyribonuclease. We suggest that this variation is a result of transformation. A second variation in colonial morphology involved differing levels of "colony opacity-associated proteins" in the outer membrane. This variation was also inhibited by the presence of deoxyribonuclease, but the genetic basis for it is not as yet clear.

It now seems to be firmly established that there is a strong correlation between the degree of virulence a particular strain of *Neisseria gonorrhoeae* displays in vivo and its colonial morphology. The T1 and T2 colony types, which predominate in recent isolates, appear to be virulent in both human volunteers and various animal models. On nonselective subculture these strains give rise to large numbers of T3 or T4 colonies, which are relatively avirulent (2, 9).

There is a considerable variation of colony types within the well-described T1, T2, T3, and T4 types, as well as other colonial morphologies which do not fit into any of the described types (8, 18). It has now been shown that a large part of this variation in colonial morphology can be correlated with specific changes in a series of outer membrane proteins (18, 19) and also with the presence or absence of pili on the cell surface (6, 8, 9, 20).

Clearly, any complete understanding of the biochemical and physiological changes affecting colonial morphology can only come by determining the genetic basis for virulence (or avirulence). Advances in this area have been hampered because the only well-defined genetic system in *N. gonorrhoeae* (transformation) is restricted to the piliated variants (17).

Despite intensive investigation, the basic mechanism(s) which allows colonial variation is still not understood. This work was undertaken in an effort to ascertain whether genetic exchange mechanisms could account for the observed high rate of colonial variation.

MATERIALS AND METHODS

Gonococcal strains. The main strain used in these experiments is 82409/55, which was obtained from A. Reyn, Copenhagen, Denmark. Strain Um03 is a derivative of 82409/55, containing the 24.5-megadalton (Mdal) plasmid (12). The colony types were maintained on a daily basis by restreaking single colonies on GC medium or were stored at −80°C in 20% (vol/vol) glycerol in the same medium.

Media and growth conditions. The solid medium used was GC medium base (Difco) supplemented with 1% (vol/vol) Kellogg supplement (11). Plates were incubated at 37°C in 6% CO_2. The liquid medium was identical to the solid medium, except that agar was omitted and 10 mM $NaHCO_3$ was added. Growth was followed in a Klett-Summerson photometer (red filter).

Chemicals. DNase I was purchased from Sigma Chemical Co., St. Louis, Mo. Both a crude DNase and a chromatographically prepared DNase were used. No difference in the results of the experiments in liquid medium was seen.

Colonial morphology. Several different laboratories have developed nomenclature systems for the variants observed in *N. gonorrhoeae* (8, 13, 18, 19). Until a uniform nomenclature system evolves, it seems appropriate in all work on colonial variation to describe the variants in terms of (i) the presence or absence of pili and (ii) the outer membrane protein composition. In accordance with this, Table 1 shows the nomenclature system used in this paper and the assumed equivalent classes in other nomenclature systems. The presence or absence of pili was scored by colonial morphology and confirmed by scanning electron microscopy (3, 6, 8, 9, 20). Colonial morphology was scored essentially by the methods of Kellogg et al. (8) and Swanson (18, 19). T1Y and T4Y variants were intermediate in coloration and opacity. The protein compositions of the outer membranes of the variants are in agreement with the assignments of Swanson (18, 19). There was no detectable difference in the outer membrane protein profiles between piliated and nonpiliated variants possessing the same coloration and opacity.

Agarose gel electrophoresis. The agarose gel electrophoresis and preparation of clear lysates were performed as described in the accompanying paper (12).

Autolysis measurements. Logarithmic cells were

harvested by centrifugation and resuspended in Tris-hydrochloride buffer (0.05 M, pH 7.2). The suspensions were incubated at 22°C. The autolysis was followed by optical density readings at 450 nm, viable count, and release of RNA. The latter was tested by withdrawing 0.5-ml samples from each culture and measuring RNA concentration by the method described by Schneider (15).

RESULTS

Single colonies of each of the colonial variants of strain 82409/55 were picked off a GC plate after 18 h of incubation, diluted, and plated to determine the rate of segregation to other colonial variants. The results (Table 2) demonstrate that this segregation tends to favor the nonpiliated colonial forms, and also those forms lacking the outer membrane proteins that have been called the "colony opacity-associated" (COA) proteins (19).

Different strains appear to have slightly different frequencies of variation, but the overall pattern of segregation appears to be the same in all the strains we have examined (data not shown). The occurrence of these colonial variants suggests that they emanated from segregating cells within the original, seemingly pure colony.

Results of an attempt to study the kinetics of the segregation of nonpiliation are shown in Fig. 1. A single T2 colony was picked off, diluted, plated on a GC plate, and incubated. Starting at 18 h, at 2-h intervals, several T2 colonies were picked off and plated to determine the fraction of nonpiliated cells within the colony. The frequency of the nonpiliated variants rose fivefold between 16 and 28.5 h, but the majority of this increase occurred after 24 h (Fig. 1). This sudden rise suggests that the frequency of variation might be increasing mainly in the stationary phase of growth. This was tested by growing a piliated T2 variant in liquid culture and measuring the frequency of variation to the nonpiliated forms in the different phases of growth. Figure 2a shows that in a culture kept in exponential growth, the frequency of nonpiliated variants rises only slightly. When the cells entered stationary phase a dramatic increase in the frequency of nonpiliated variants was observed (Fig. 2b). As gonococci enter the stationary phase of growth, they have a pronounced tendency to autolyse (5). One of the most obvious effects of this autolysis is a large release of DNA into the culture medium. If this DNA release

TABLE 1. *Nomenclature system for colonial variants*

Colony type	Different nomenclature systems				
	Kellogg et al. (8)	Swanson (18, 19)[a]			Penn et al. (13)
T1	T1	p^+	Tr	XLi	SH
T1Y	T1	p^+	Op/Tr	Li	SH
T2	T2	p^{++}	Op	Da	DH
T3	T3	p^-	Op	Da	NH
T4Y	T4	p^-	Op/Tr	Li	NH
T4	T4	p^-	Tr	XLi	NH

[a] p refers to the state of piliation (18).

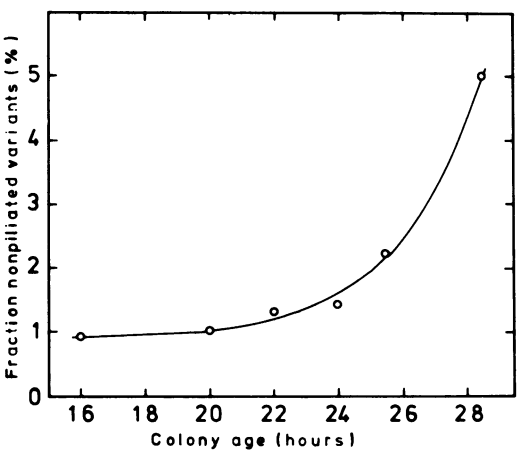

FIG. 1. *Effect of colony age on segregation to nonpiliation. The frequency of segregation to nonpiliation within individual T2 colonies was determined as described in the text.*

TABLE 2. *Frequency of colonial variation in N. gonorrhoeae strain 82409/55*

Resulting colony variant[a]	Colonial variant plated[a]											
	T1 (p^+)		T1Y (p^+)		T2 (p^{++})		T3 (p^-)		T4Y (p^-)		T4 (p^-)	
	No.	%	No.	%	No.	%	No.	%	No.	%	No.	%
T1 (p^+)	986	98.2	34	1.6								
T1Y (p^+)	6	0.6	2,086	96.7	14	1.4						
T2 (p^{++})	6	0.6	14	0.6	1,011	98.2						
T3 (p^-)					4	0.4	998	98.2	21	1.0	2	0.2
T4Y (p^-)			24	1.1			18	1.8	1,975	97.4	6	0.6
T4 (p^-)	6	0.6							32	1.6	1,022	99.2

[a] p^+ or p^{++}, Piliated variants; p^-, nonpiliated variants (18).

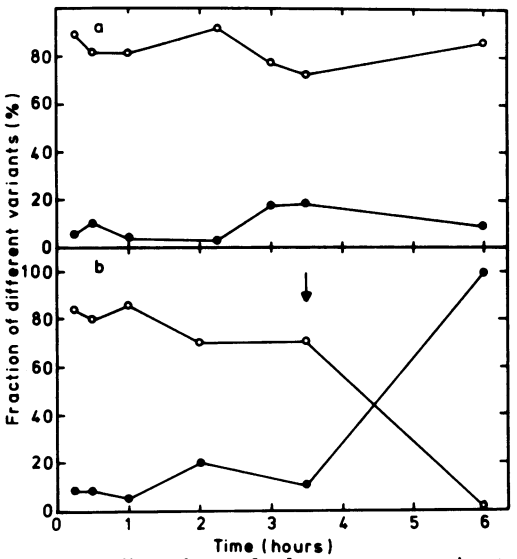

FIG. 2. *Effect of growth phase on segregation to nonpiliation. (a) Frequency of piliated (T2)* (○) *and nonpiliated* (●) *variants in a culture kept in the exponential phase of growth by periodic dilution. (b) The same frequencies for a parallel culture which was allowed to enter stationary phase. The arrow marks the cessation of exponential growth.*

was important in determining colonial variation, one might expect that the segregation to other colonial variants would be sensitive to the action of DNase.

The experiment shown in Fig. 3 demonstrates the effect of DNase on segregation of a piliated strain (T2) in liquid medium. Most of the colonial variation (both to nonpiliated forms and to those lacking the COA proteins) appears after active growth of the culture has ceased, and this variation appears to be DNase sensitive. It is interesting to note that the appearance of T4 variants seems to be delayed when compared with the appearance of the T1 and T3 colony types. This suggests that two separate events are involved in the change from the T2 to the T4 colony type. The addition of DNase did not affect onset of autolysis, as verified by viable count measurements (data not shown).

The presence of DNA in culture supernatants can be demonstrated directly. We have routinely found such supernatants to contain between 1 and 2 μg of DNA per ml (as determined spectrophotometrically). Furthermore, this DNA has a high molecular weight. When the DNA from such a supernatant was concentrated by ethanol precipitation and loaded onto an agarose gel, a

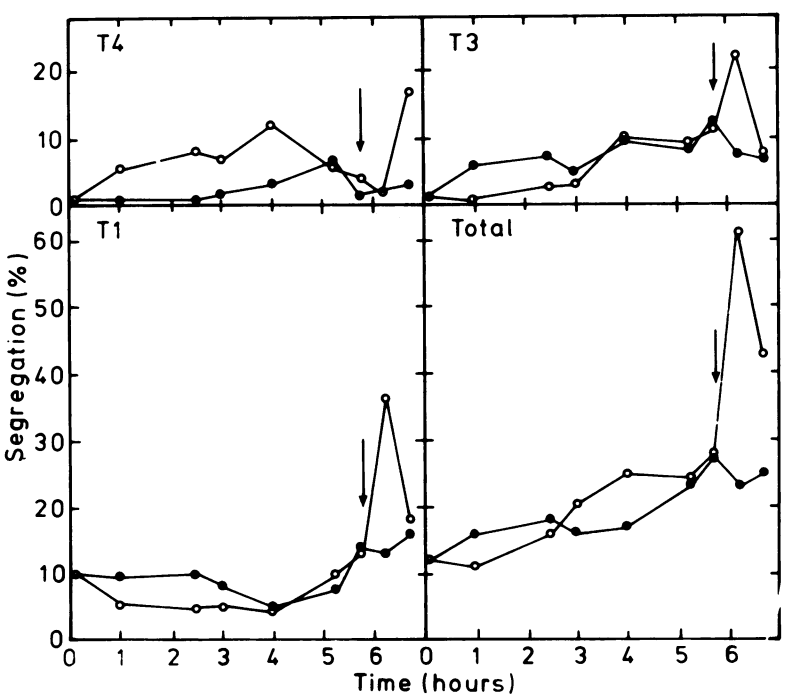

FIG. 3. *Effect of DNase on the segregation of T2 variants to other colony types in liquid media. T2 colony variants were grown in liquid media in the presence or absence of DNase (10 μg/ml). Samples were withdrawn at indicated times and spread on GC plates. After incubation the percentage of each colony variant was determined. The arrows indicate the onset of autolysis. Symbols:* (○) *variants in an untreated culture;* (●) *variants in a DNase-treated culture.*

broad high-molecular-weight DNA band was evident at a position identical to that observed for chromosomal DNA from a cleared lysate (Fig. 4).

The addition of DNase can be seen to have a positive effect, as shown in Fig. 3. In addition, however, it has been possible to demonstrate directly that DNase is active against the DNA present in the media, at the concentrations and under the conditions used here. Figure 4 demonstrates that when a DNase solution (50 μg/ml) is incubated at 37°C for periods up to 4 h and then added to DNA concentrated from a culture supernatant, it can effectively degrade this DNA. It should be emphasized that in this concentrated sample the DNA is approximately 25 times more concentrated than it is in a normal culture supernatant, indicating that excess DNase activity is still present after 4 h of incubation at 37°C. The results shown in Fig. 4 also demonstrate that there is apparently no deficiency of Mg^{2+} under the conditions used here. We have also shown that DNase solutions at lower concentrations (e.g., 10 μg/ml) retain activity after 4 h at 37°C (data not shown). In all

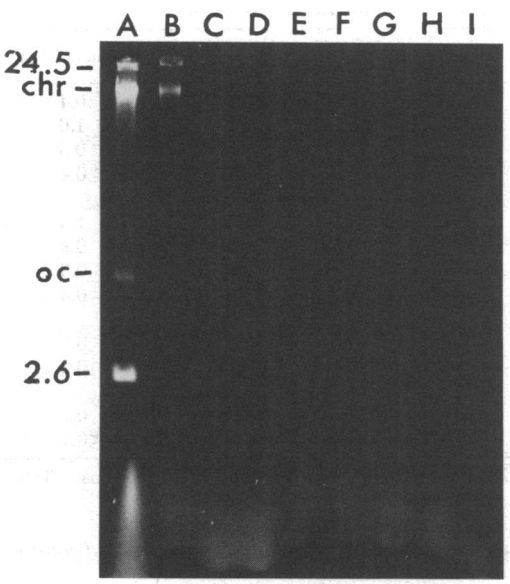

FIG. 4. *Effect of DNase on high-molecular-weight DNA in a culture supernatant. Slot A shows a cleared lysate of a strain containing the 2.6- and 24.5-Mdal plasmids, and B shows the precipitated DNA from supernatant. The other slots show precipitated supernatant DNA incubated for 1 h at 37°C with DNase: 10 μg/ml (C); 50 μg/ml (D); and 50 μg/ml with 2 mM Mg^{2+} added (E). In slots F to I the DNase solution (50 μg/ml) was incubated at 37°C for 1, 2, 3, and 4 h, respectively, before addition to the supernatant DNA. chr, Chromosome; oc, open circular form of the 2.4-Mdal plasmid.*

experiments reported here, fresh DNase was added at 2.5-h intervals.

The DNase sensitivity of colonial variation can also be observed on solid media. If individual colonies of the different colony types are restreaked at 24-h intervals on GC plates, or on GC plates containing 10 μg of DNase per ml, a distinct difference is seen in the frequency of colonial variation (Table 3). In this particular experiment, the T1 variant showed a low rate of segregation to the nonpiliated forms. The effect of DNase is therefore not as obvious as it is in the case of the T2 variant. It should be noted that this difference is only obvious if the colonies are restreaked immediately onto solid media. If the colony is picked off the plate and diluted in liquid medium and a sample is spread to give single colonies, the total variation observed is much less (whether or not DNase is present), and the differences are not as obvious. Presumably this is because the resuspension of the colony in liquid medium effectively dilutes the extracellular DNA concentration.

From the results presented in Table 3, it can be seen that the presence of DNase has an effect not only on variations in the state of piliation, but also on variations in the amount of COA proteins present, in both the piliated and the nonpiliated variants. It should be noted here that the crude preparation of DNase used in the solid medium experiments appeared to affect the coloration and opacity of the colonies. When T3 or T2 colonies were restreaked on plates containing DNase they appeared to be slightly less opaque and less colored than the same colonies growing on plates without DNase. We have interpreted this to be the result of a contaminating proteolytic effect in the DNase preparation, causing the loss of some of the COA proteins and resulting in a colony that appears to be intermediate between the T2 and T1Y or T3 and T4Y types. Swanson (19) has shown that the COA proteins are extremely sensitive to the effects of certain proteolytic enzymes. Support for this interpretation of our results comes from the observation that colonies with altered coloration and opacity immediately revert to the original (T2 or T3) colony type when plated on media lacking DNase. Although this effect may have caused some errors in our estimation of the rate of segregation, in the presence of DNase, from forms possessing the COA proteins to forms lacking them, it cannot have had any effect on our estimation of the frequency of variation in the opposite direction or on the observed rates for the appearance of nonpiliated variants.

Recently, we have shown that the presence in nonpiliated variants of a 24.5-Mdal plasmid confers competence in transformation (12). It was

therefore of interest to determine whether the presence of this plasmid has any effect on the frequency of colonial variation in the nonpiliated colony types. The results presented in Table 4 demonstrate that nonpiliated strains carrying this plasmid show a considerable increase in the frequency of segregation, on solid media, to other colony types. As might be expected, this variation is still sensitive to the action of DNase. Figure 5 shows the results of an experiment in liquid media. Again, the presence of the plasmid appears to result in an increase in the rate of segregation to other variants.

The above experiments strongly imply that autolysis plays an important role in colonial variation. It was therefore important to determine whether any differences in the autolytic processes could be detected between the different colonial variants. Figure 6 demonstrates that the piliated (in this case T1) variant is delayed in its lysis under conditions where active growth is no longer possible. We could detect no significant difference in the kinetics of lysis in the different nonpiliated variants (data not shown).

DISCUSSION

Although some data on the differences between the different colonial variants of *N. gonorrhoeae* have recently begun to accumulate, it has not been apparent until now what the basic mechanisms for such variability are. The widespread variations in colonial morphology noted here, and the relatively high rates of segregation from one colony morphology to another, confirm the results noted by many others (7, 9). Most of these variations seem to occur after active growth, either in individual colonies or in liquid cultures, has ceased. This point has also been noted by others (10), but no explanations for this observation have been forthcoming. To elucidate the mechanisms for these variations, we initially tried to ascertain whether they might have a genetic basis. It seems logical to first determine whether a genetic system is operating before embarking on a search for a physiological basis for the high frequencies observed. In addition, the relative stability of the various segregants, when replated on the same media, sug-

TABLE 3. *Effect of DNase on the frequency of colonial variation on solid media*

Colony type plated	Presence of DNase	Total colonies counted	Changes observed[a]	Variants counted	Frequency (%)
T1	−	1,289	$Pili^+ \rightarrow Pili^-$	5	0.4
			$COA^- \rightarrow COA^+$	21	1.6
	+	1,167	$Pili^+ \rightarrow Pili^-$	4	0.3
			$COA^- \rightarrow COA^+$	5	0.4
T2	−	1,309	$Pili^+ \rightarrow Pili^-$	17	1.3
			$COA^+ \rightarrow COA^-$	12	0.9
	+	1,177	$Pili^+ \rightarrow Pili^-$	4	0.3
			$COA^+ \rightarrow COA^-$	4	0.3
T3	−	1,091	$COA^+ \rightarrow COA^-$	10	0.9
	+	1,037	$COA^+ \rightarrow COA^-$	2	0.2
T4	−	1,080	$COA^- \rightarrow COA^+$	8	0.7
	+	2,006	$COA^- \rightarrow COA^+$	0	<0.1

[a] $Pili^+$, piliated variant; $Pili^-$, nonpiliated variant. COA^+, variants possessing the COA proteins; COA^-, variants lacking COA proteins.

TABLE 4. *Effect of the presence of the 24.5-Mdal plasmid on colonial variation among nonpiliated colony types on solid media*

Strain	24.5-Mdal plasmid	Presence of DNase	Colony type plated	Total colonies counted	Change observed[a]	Variants counted	Frequency (%)
82409/55	−	−	T3	1,091	$COA^+ \rightarrow COA^-$	10	0.9
			T4	1,080	$COA^- \rightarrow COA^+$	8	0.7
Um03	+	−	T3	1,161	$COA^+ \rightarrow COA^-$	19	1.6
			T4	1,127	$COA^- \rightarrow COA^+$	24	2.1
		+	T3	1,383	$COA^+ \rightarrow COA^-$	11	0.8
			T4	1,023	$COA^- \rightarrow COA^+$	15	1.5

[a] COA^+, Variants possessing the COA proteins; COA^-, variants lacking the COA proteins.

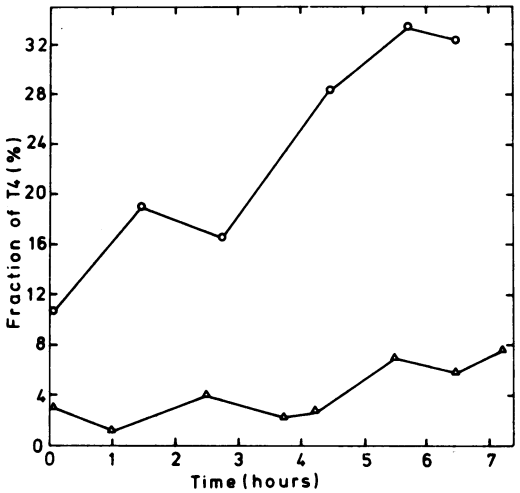

FIG. 5. *Effect of the presence of the 24.5-Mdal plasmid on the segregation of T3 variants to other colony types in liquid media. A T3 variant of strain 82409/55 (△) and a T3 variant of strain Um03 (○) (a derivative of 82409/55 carrying the 24.5-Mdal plasmid) were grown in GC liquid medium. Samples were withdrawn at the indicated times and spread on GC plates to determine the frequency of the different colony types.*

gests a genetic basis for colonial variation.

The frequencies of colonial variations observed are too high (often >1%) to be explained by a simple selection for mutants. The three known mechanisms for the exchange of genetic material, transformation, conjugation, and transduction, can all operate with an efficiency that could lead to recombinant formation at the frequencies observed for colonial variation. No known transducing bacteriophages occur in gonococci, but both transformation and conjugation have been shown to occur (12, 14, 16, 17). The fact that colonial variation obviously occurs in the absence of the 24.5-Mdal plasmid and the DNA sensitivity of this variation seem to rule out any role for conjugation in this process.

Could transformation therefore account for the colonial variation observed when *N. gonorrhoeae* is grown in vitro? It has been known for some time that the piliated variants are competent for transformation and the nonpiliated variants are not (17). We have demonstrated that in a mixed culture, autolysis of the nonpiliated variants begins distinctly before that of the piliated variants. As a result of this early lysis of nonpiliated cells, there is a large release of DNA. If this DNA then transformed the piliated cells

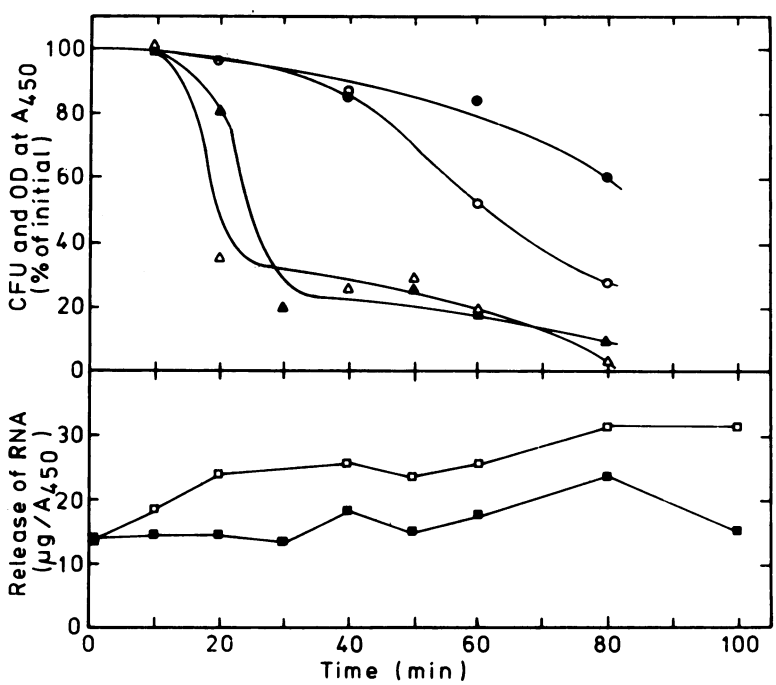

FIG. 6. *T1 and T4 colony variants of strain 82409/55 pregrown in GC medium were resuspended in a Tris-hydrochloride buffer (0.05 M, pH 7.2) and incubated at 22°C. Samples were withdrawn at the indicated time intervals, and the optical density (OD) at 450 nm (A_{450}) was measured. The samples were also diluted and plated to determine colony-forming units (CFU), and the release of RNA was measured as described in the text. Symbols: (●) OD, T1; (○) OD, T4; (▲) CFU, T1; (△) CFU, T4; (■) RNA/A_{450}, T1; (□) RNA/A_{450}, T4.*

with a high efficiency, there would be a rapid increase in the incidence of nonpiliated cells several hours later. During this period, which is needed to allow time for the expression of the nonpiliated state, the piliated cells have also begun to autolyse. Thus, the sudden emergence of a large number of nonpiliated cells has an increased effect on the fraction of nonpiliated cells in the culture. Figure 7 shows the predicted result for a theoretical experiment, in which it was assumed that a culture of piliated variants, containing 5% nonpiliated types, had ceased active growth. It was calculated using an initial cell density of 10^9 cells per ml and assuming that the nonpiliated cells begin to release their DNA 45 min before the piliated variants, although the rate of loss of colony-forming ability was the same for both variants (half-life = 15 min). These assumptions were derived from our data on the release of RNA and loss of viability, respectively (Fig. 6). The efficiency of transformation was assumed to be 10^{-5} per recipient cell per 30 min for any particular marker, and it was assumed that it takes 2.5 h for the nonpiliated state to be expressed. From the curve shown in Fig. 7, it is apparent that under these conditions, there should be a noticeable increase in the percentage of nonpiliated variants as the culture starts to lyse.

Our data demonstrate a similar result. We find that the percentage of nonpiliated variants remains relatively constant until the culture ceases active growth, when there is a dramatic increase in the frequency of nonpiliated variants. If this increase is solely the result of transformation, it should be sensitive to the action of DNase. The results presented above clearly demonstrate that this is the case. Thus, we conclude that at least a large part of the variations observed in the state of piliation can be accounted for solely by transformation. We interpret the subsequent partial decrease in the frequency of variation (e.g., see Fig. 3) to be the result of "chasing" of variant DNA by excess DNA from the original colony type.

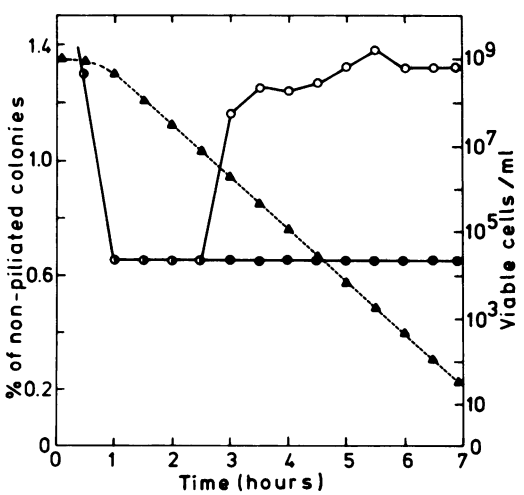

FIG. 7. *Calculated result of a theoretical experiment in which the percentage of nonpiliated variants is monitored after active growth of a piliated variant, grown in the presence (●) or absence (○) of DNase, has ceased. There were assumed to be 5% nonpiliated variants present at the time growth ceased. For other assumptions, see text. The total number of viable cells per milliliter in the entire culture (▲) is also shown.*

The explanation for the segregation to colonial forms with different amounts of the COA proteins is less obvious. Although this variation within the piliated forms could be explained by transformation, it occurs with approximately the same frequency in nonpiliated variants, which are normally incompetent for transformation by chromosomal DNA. This argues strongly that this variation is not mediated by transformation. Yet, the process is clearly DNase sensitive, which supports the involvement of extracellular DNA. In addition, we have recently shown that the presence of the 24.5-Mdal plasmid confers competence for transformation of chromosomal markers on nonpiliated variants (12). We have shown that the presence of this plasmid also dramatically increases the frequency of colonial variation among the nonpiliated forms and that this increase is DNase sensitive. This again supports the idea that transformation is involved.

One possible explanation for this apparent contradiction could be that the genetic information for the COA proteins is plasmid coded and that this plasmid is transformed with a frequency much higher than that observed for chromosomal markers. There are two pieces of evidence that make this hypothesis unlikely. First, there exist several plasmid-free strains which show variations in the amounts of COA proteins (unpublished data). Second, Elwell and Falkow (4) have reported that, at least for the penicillin resistance plasmid, the efficiency of transformation of a plasmid marker (ampicillin resistance) was much lower than that for many chromosomal markers. Clearly, any understanding of the genetic basis for the variation in the COA proteins must await the results of future work. Our results suggest that the loss of piliation and the loss of the COA proteins are two separate events. The observation that it takes longer to lose both characters than either single character (Fig. 3) is consistent with this view.

Another interesting observation is the fact that strains carrying the 24.5-Mdal plasmid show

a frequency of variation that appears to favor the presence of the COA proteins (Table 4); i.e., the frequency for COA^- to COA^+, unlike the normal case, is greater than the rate for COA^+ to COA^-.

Recently, it has been reported that it is possible to transform nonpiliated cells to piliation with DNA from piliated variants, and also to an unusual piliated colonial form with DNase-treated DNA (1). We have not been able to confirm these results, and note that others have also been unable to repeat these experiments, even when utilizing the same strains (17). We feel that the results obtained with DNase-treated DNA may perhaps be explained by the contaminating proteolytic effect we have observed in some relatively crude DNase preparations. We have noted that the "wrinkled" variants observed by these investigators are relatively resistant to the proteolytic effects and, therefore, become readily noticeable among the background of partially colored colonies (data not shown).

In summary, we suggest that at least a large part of the colonial variation observed in *N. gonorrhoeae* is caused by the efficent mechanisms for the exchange of genetic information.

ACKNOWLEDGMENTS

J.D. is the recipient of a Visiting Scientist Fellowship of the Swedish Medical Research Council. This work was supported by a grant (Dnr 4769) from the Swedish Medical Research Council.

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