SHORT COMMUNICATION

Degrees of *O*-Acetylation and Cross-linking of the Peptidoglycan of *Neisseria gonorrhoeae* during Growth

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The progress of incorporation of radioactive glucosamine into the *O*-acetylated and non-*O*-acetylated sub-units of the insoluble peptidoglycan of growing *Neisseria gonorrhoeae* was examined. More than 80% of the final degree of cross-linking was achieved within 3 min; the remainder of the process took much longer. Rapid *O*-acetylation occupied up to 10 min, at which time only about 65% of the maximum value had been reached. There was thus evidence for maturation of peptidoglycan both in regard to cross-linking and to *O*-acetylation.

INTRODUCTION

Recently we established that the peptidoglycan of *Neisseria gonorrhoeae* is partially *O*-acetylated (Blundell *et al.*, 1980) and that growing bacteria respond to low concentrations of β -lactam antibiotics by an overall increase in peptidoglycan coupled with decreased *O*-acetylation and little change in cross-linking (Blundell & Perkins, 1981). In those experiments the bacteria were grown in medium containing radioactive glucosamine, which labelled both amino sugars of the peptidoglycan, and samples were taken for analysis after about two generations of growth. Digestion with muramidase B from *Chalaropsis* sp. (Hash & Rothlauf, 1967) yielded monomer, dimer and oligomer units of peptidoglycan, which were separated by TLC (Martin & Gmeiner, 1979). Measurement of radioactivity in the separated fractions allowed the degrees of cross-linking and of *O*-acetylation to be measured (Blundell & Perkins, 1981).

The present work examined the fate in terms of cross-linking and *O*-acetylation of a short pulse of radioactive glucosamine. It was not possible to 'chase' this with an excess of unlabelled precursor because preliminary experiments showed that growth of *N. gonorrhoeae* strain IL260 was inhibited by more than about 35 μ M-glucosamine. Although addition of glucose immediately prevented the uptake of glucosamine, this method was avoided because of possible difficulties in interpretation after a change of carbon source.

METHODS

Neisseria gonorrhoeae strain IL260, stored as described earlier, was grown on plates overnight and used to inoculate 60 ml samples of supplemented proteose-peptone with pyruvate as carbon source (Blundell & Perkins, 1981). The cultures were incubated with shaking at 37 °C until growth had reached $A_{675} = 0.3$, when D-[1- 14 C]glucosamine hydrochloride [54 μ Ci μ mol $^{-1}$ (2.0 MBq μ mol $^{-1}$); Amersham] was added to a final concentration of 1.25 μ Ci ml $^{-1}$ and shaking was continued. After 10 min, a 20 ml sample was removed and centrifuged (2750 g, 1.25 min, bench centrifuge). The pellet, freed as far as possible from medium, was resuspended in 25 ml fresh medium, prewarmed and equilibrated with 5% CO $_2$ in air. Incubation of both this culture and the parent one was continued for a further 1.25 generations. Both grew exponentially throughout the experiment; the resuspended sample grew immediately without lag.

Duplicate 2 ml samples were taken at intervals, added to an equal volume of 10% (w/v) SDS (Sigma) and heated at 100 °C for 20 min (Blundell *et al.*, 1980). The incorporation of radioactivity was measured by filtering a 50 μ l

sample on glass microfibre discs (GF/C, Whatman), washing, drying and counting (Blundell & Perkins, 1981). The remainder of the 2 ml sample was used for isolation of SDS-insoluble material as before (Blundell *et al.*, 1980) and the resulting peptidoglycan was digested with *Chalaropsis* muramidase B (given by Dr J. B. Ward) and prepared for TLC in isobutyric acid/1 M-ammonia (5:3, v/v) (Blundell & Perkins, 1981). Samples of digest equivalent to about 40000 d.p.m. were used for TLC and the radioactive spots were located by autoradiography, cut from the TLC and counted in non-aqueous scintillant. The peptidoglycan components of the *Chalaropsis* muramidase digest were identified by comparison with markers that had previously been characterized as monomer (i.e. disaccharide-peptide), *O*-acetylmonomer, dimer (bis-disaccharide-cross-linked peptide), mono-*O*-acetyldimer, di-*O*-acetyldimer and oligomers (i.e. trimers and greater, in which the *O*-acetylated components could not be distinguished).

RESULTS

The progress of peptidoglycan synthesis by growing *N. gonorrhoeae* was followed by observing into which of the *Chalaropsis* muramidase products the radioactivity added to the culture as glucosamine became incorporated. In parallel experiments, the bacteria were either left to grow in continuous contact with the labelled precursor, or centrifuged after 10 min and resuspended in fresh medium. In the former case, incorporation into the SDS-insoluble material continued essentially linearly for the whole 90 min period; in the latter, little further increase occurred during the period after resuspension. The degrees of *O*-acetylation of total monomer and dimer fractions reported in Fig. 1 (a) and the degree of cross-linking of the labelled peptidoglycan (Fig. 1 b) were calculated (Blundell & Perkins, 1981). Already at 7 min (one-tenth of a generation time in this experiment) 29% of the peptide chains had become cross-linked, representing 84% of the final value achieved by the pulse sample (35%). In other experiments, even earlier samples (3 min) still had about 29% cross-linking. As might be expected, continuous labelling yielded slightly lower values, presumably because the overall label included some that was relatively new and some old.

Fig. 1. Progress of cross-linking and *O*-acetylation of SDS-insoluble peptidoglycan during growth. Radioactive glucosamine was added at time 0. After 10 min, a sample was removed, centrifuged, resuspended in fresh medium and re-incubated (open symbols) while the incubation of the original culture continued (closed symbols). (a) The degree of *O*-acetylation of isolated monomer (○, ●) and dimer (□, ■) units. Values represent the percentage of total disaccharide units in the fraction that were *O*-acetylated. (b) The degree of cross-linkage as defined by Blundell & Perkins (1981) (△, ▲). (c) Degrees of *O*-acetylation of the monomer (●—●) and dimer (■—■) fractions: mean values from many separate continuous labelling experiments. The bars indicate s.d. but some are omitted for the sake of clarity. (d) Proportion of total radioactivity found in monomer (○), dimer (□) and oligomer (▽) fractions in the sample labelled for only 10 min.

O-Acetylation of the newly incorporated peptidoglycan was a much slower process (Fig. 1*a*). At 7 min, the monomer fraction was only 40% *O*-acetylated compared with a final value for the pulse experiment of 60%, although by 30 min the degree of *O*-acetylation of monomer approached the upper value. The very early stages of *O*-acetylation of monomer can be seen in Fig. 1*c*), which is a composite graph prepared from a large number of different experiments in which radioactive glucosamine was present throughout. At 3 min, only 26% of monomer had become acetylated and an extremely rapid rise to 40% at 10 min followed. A lower rate of *O*-acetylation followed until a maximum of 60% was achieved at about 45 to 55 min. As in the experiment of Fig. 1*a*), there was some evidence that the degree of *O*-acetylation of monomer decreased in the final 30 min out of 90 min total.

The *O*-acetylation of the dimer fraction took a similar time to develop and in the pulse experiment there was clear evidence for continuing *O*-acetylation even in the second 30 min after addition of label. Continuous labelling produced somewhat lower final values for *O*-acetylation, no doubt related to the origin and fate of the fragments of peptidoglycan found at any one time in this fraction. The degree of *O*-acetylation of dimer recorded for a whole series of experiments (Fig. 1*c*) followed a somewhat similar pattern to that of the monomer. Here no distinction could be made, for instance, between mono-*O*-acetylated dimer in which the non-*O*-acetylated monomer unit was labelled and one in which the new unit was the *O*-acetylated portion.

The way in which the labelling of the monomer, dimer and oligomer fractions changed with time after label had been added for 10 min and then removed is shown in Fig. 1*d*). Monomers, which at first represented 42% of the whole, declined after 90 min to 32%. The proportion of dimers changed but little from its initial value of 40% and it was the oligomers that increased to compensate for the disappearance of monomers.

DISCUSSION

The results show that during the synthesis of peptidoglycan in growing cultures of *N. gonorrhoeae*, the bulk of the cross-linking occurred very rapidly (84% within 3 min) while the remainder extended over approximately half a generation time. This is very similar to the maturation process described in *Escherichia coli* by de Pedro & Schwarz (1981) and in *Proteus mirabilis* by Gmeiner & Kroll (1981). In *E. coli* the maturation took from 0.5 to 1 generation time and involved the formation of new dimer units. In *N. gonorrhoeae* the main effective change was an increase in oligomers, although of course dimers were labelled and must have been intermediates for the formation of oligomers. De Pedro & Schwarz (1981) had evidence that penicillin-binding protein 4 of *E. coli* was involved in the secondary cross-linking. Three penicillin-binding proteins are known for *N. gonorrhoeae* (Dougherty *et al.*, 1980; Barbour, 1981), but at present we have no evidence for their relationship to the maturation of peptidoglycan.

The *O*-acetylation of peptidoglycan in *N. gonorrhoeae* was a slower process than cross-linking, indicating that sub-units already incorporated into the pre-existing peptidoglycan must then undergo acetylation. In general outline this matches the observations of Gmeiner & Kroll (1981) for *P. mirabilis*. These authors produced some evidence that new non-*O*-acetylated monomer units were preferentially cross-linked to previously *O*-acetylated monomer units to yield mono-*O*-acetylated dimers, but at the same time some preformed dimer units were later mono-*O*-acetylated. This complicated picture will need to be resolved for *N. gonorrhoeae* as well as for *P. mirabilis* and *E. coli*.

Our results also showed (Fig. 1*b, c*) that the initial very rapid period of cross-linking (up to 3 to 10 min) was roughly paralleled by a very rapid period of *O*-acetylation (up to 10 min for the monomer or 5 min for the dimer fraction), although a lower proportion of the final value was reached. The slow decline of *O*-acetylation from both the monomer and the dimer fractions during the 60 to 90 min period could indicate either a direct loss of *O*-acetyl groups by a deacetylase, or a preferential transfer of *O*-acetylated units into the oligomer fraction, where our present methods were unable to detect them.

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