Lack of inhibition by colicin M suggests bactoprenol independence of MDO biosynthesis

Robin E. Harkness, Waltraud Fiedler and Volkmar Braun

Mikrobiologie II, Universität Tübingen, D-7400 Tübingen, FRG

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Biosynthesis of membrane-derived oligosaccharides (MDO), located in the periplasmic space of *Escherichia coli*, was not inhibited by colicin M, an inhibitor of bactoprenyl phosphate regeneration. This result suggests that bactoprenol does not serve as a lipid carrier of MDO oligosaccharides across the cytoplasmic membrane.

Membrane-derived oligosaccharide; Biosynthesis; Bactoprenol; Colicin M; (*Escherichia coli*)

1. INTRODUCTION

Membrane-derived oligosaccharides (MDO) are located in the periplasmic space between the outer and the cytoplasmic membrane of *Escherichia coli* and other gram-negative bacteria [1]. They are synthesized when the external osmolarity is low and serve to counterbalance the internal osmotic pressure [2,3]. Mutants unable to synthesize MDO are impaired in chemotactic response (motility), show a reduced number of flagella, and an enhanced synthesis of the OmpC outer membrane porin [4]. MDO are composed of 8–10 glucose units per molecule, which are linked by β 1–2 and β 1–6 bonds [5]. The highly branched glucan backbone is substituted with *sn*-1-phosphoglycerol, phosphoethanolamine, and *O*-succinyl ester residues [6].

Biosynthesis of MDO is initiated by transfer of glucose from UDP-glucose onto a carrier, and subsequent elongation of the glucan chain through a glucosyltransferase system [7]. Octyl-β-D-glucopyranoside can serve as an artificial acceptor in the transglucosylation reaction suggesting a physiological carrier with a hydrocarbon chain. C_{55}-undecaprenol (bactoprenol) serves as a lipid carrier for the translocation of oligosaccharide precursor molecules from the cytoplasm across the cytoplasmic membrane. This has been shown for the biosynthesis of murein (peptidoglycan) [8], the O-antigen portion of lipopolysaccharide [9], teichoic acids [10], and K1 capsular polysaccharides [11]. Upon transfer of the oligosaccharides to periplasmic acceptors, bactoprenyl pyrophosphate is released and reenters the biosynthetic cycle as bactoprenyl monophosphate. Cleavage of the pyrophosphate bond is accomplished by a membrane-bound phosphatase [12]. Colicin M interferes with the regeneration of bactoprenyl phosphate, and in this way inhibits murein [13] and O-antigen biosynthesis [14]. Inhibition of murein synthesis results in lysis of cells which is the primary cause of cell killing by this bacterial toxin [15,16].

In this study we employed colicin M as a tool to study the unsettled question of the involvement of bactoprenol in MDO biosynthesis [17]. We found no inhibition of MDO biosynthesis by colicin M which makes it unlikely that bactoprenol acts as lipid carrier in MDO biosynthesis.

Correspondence address: V. Braun, Mikrobiologie II, Auf der Morgenstelle 28, Universität Tübingen, D-7400 Tübingen, FRG

2. EXPERIMENTAL

2.1. *Bacterial strains*

The following *E. coli* K-12 strains were used: MA1008 (*lacZ*43 *pyrc*46 *thi*-1 *relA*1 *spoT*1) [18], TA1008 (*mdoA*1 *pyrC*$^+$ MA1008 [4], and EH3247 (*glmS ilv rpsE thi thr* [19]. The latter strain was provided by Joachim Höltje, Max-Planck-Institut für Entwicklungsbiologie, Tübingen. Strain EH3247 is auxotrophic for GlcN or GlcNAc as it is defective in L-glutamine: D-fructose-6-phosphate amino transferase (EC 2.6.1.16). We were not able to make this strain defective in MDO biosynthesis (*mdo*) since it already exhibited the two screenable phenotypes, loss of motility and slimy colonies on solid media, associated with the *mdo* genotype.

2.2. *Growth conditions*

Bacteria were maintained in TY medium (0.8% tryptone, 0.5% yeast extract, 0.25% NaCl) supplemented with 100 μg GlcNAc/ml or 100 μg uracil/ml as required. To induce MDO synthesis cells were grown in low osmotic strength medium (peptone medium) containing 0.5% peptone and 10 μg thiamine/ml supplemented with GlcNAc or uracil as required. Cultures were incubated at 30°C with aeration.

2.3. *Radiolabelling cells*

At selected times, 1 ml aliquots were taken from actively growing cultures (in peptone medium) and pulse-labelled at 30°C for 3 min

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with 9 kBq (0.9 nmol) D-[U-^{14}C]glucose (10.0 GBq/mmol; Amersham International). After labelling, cells were harvested by centrifugation and suspended in 40 μl H_2O and 10 μl isobutyric acid. Labelled products were separated by descending paper chromatography in an isobutyric acid/1 M NH_4OH (5:3) solvent system as described elsewhere [13]. Distribution of radioactivity on chromatograms was determined by liquid scintillation counting 1 cm segments of paper cut along the length of the chromatogram, using a toluene-based scintillation cocktail.

2.4. *Other procedures*

MDO used as a chromatographic marker was isolated as previously described [4,20] from strain MA1008 labelled with 37 kBq (57 pmol D-[2-^{3}H]glucose (Amersham International)/ml culture. Colicin M was isolated as described [21]. Colicin-treated cultures were given pure colicin M to a final concentration of 0.5 μg/ml. Protein was determined by the method of Bradford [22].

3. RESULTS AND DISCUSSION

The column-chromatographic method hitherto used to separate MDO from other components present in the 50% ethanolic cell extracts was too labor-intensive to study the time course of MDO biosynthesis in the presence and absence of a potential inhibitor (colicin M). Therefore, a paper chromatographic method with great resolving power was employed for MDO identification and quantitation. [^{3}H]glucose-labelled MDO purified by column chromatography separated on paper between R_f 0.11 and 0.19 with a peak at 0.15. The glucose substrate migrated further separating at an R_f of 0.49 (fig.1A). Glucose pulse-labelled whole cells of strains MA1008 and TA1008 subjected to chromatographic separation yielded 5 major radiolabelled peaks (fig.1B). One of these peaks in the MA1008 profile migrated at the same R_f as the MDO standard. This peak was missing from the chromatographic profile of the *mdo* mutant (strain TA1008). Therefore, MDO can be separated from other labelled cellular components by this chromatographic system. The identity of the other peaks is not known. It should be possible to obtain greater resolution of the MDO peak from the neighbouring large peak (R_f 0.23), should this be desired, by developing the chromatogram for longer periods of time (48–72 h instead of 24 h as was done in this study).

Although biosynthetic precursors of murein biosynthesis (UDP MurNAc peptides) separate between R_f 0.1–0.2 with this system [23,24] did not interfere with the identification of MDO, probably because their amount was much lower than that of MDO. Nevertheless, we used strain EH3247 for the quantitative determination of MDO as this strain is unable to metabolize glucose into GlcNAc which is contained as such and as *N*-acetylmuramic acid derivative in the murein glycan chains. Strain EH3247 showed the same

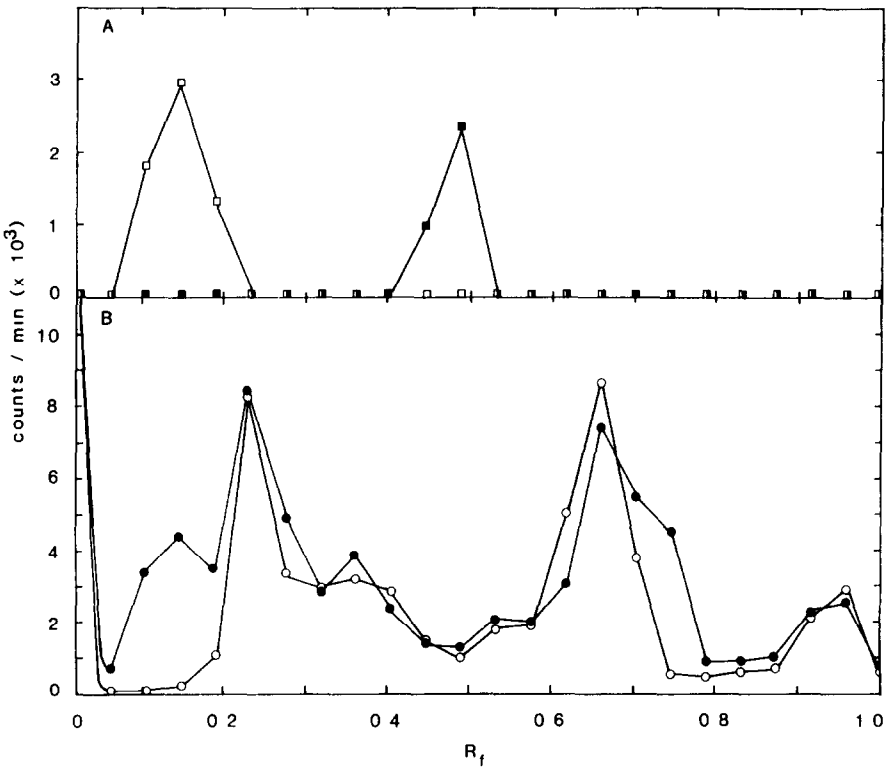

Fig.1. Separation pattern of MDO and glucose (A) and whole cells of strains MA1008 (*mdo*$^+$) and TA1008 (*mdo*) (B) following paper chromatography. Samples were radiolabelled and prepared as described in section 2. Developed chromatograms were cut into 1 cm segments starting at the origin (R_f 0) and ending at the solvent front (R_f 1.0). Radioactivity of each segment was determined by liquid scintillation counting. Symbols used: ■, [^{3}H]glucose labelled MDO; ○ ■, [^{3}H]- and [^{14}C]glucose; ●, [^{14}C]glucose labelled MA1008; ○, [^{14}C]glucose labelled TA1008.

radioactivity profile when labelled with [^{14}C]glucose as strain MA1008 (data not shown).

Strain EH3247 grown in peptone medium lysed approximately 20 min after colicin M addition (fig.2). Therefore, the strain showed a normal sensitivity to colicin M also in the low osmotic strength medium (i.e., slow growth conditions) required for maximal induction of MDO synthesis.

Quantitation of MDO synthesized following pulse-labelling of strain EH3247 which had been treated with colicin M, showed no inhibition of MDO synthesis (table 1). Synthesis continued up to the point of lysis and then started to decrease. Residual synthetic activity measured during culture lysis was probably due to cells which had not yet lysed.

By comparison synthesis of murein and O-antigen were inhibited within the first 5 min of colicin treatment (i.e., well before culture lysis) [13,14].

If synthesis of MDO requires the bactoprenyl carrier lipid to transfer the polyglucose chains through the cytoplasmic membrane into the periplasm, as has been predicted [7,25], then a rapid inhibition of MDO synthesis would have been expected following colicin M treatment as is observed with peptidoglycan and O-antigen synthesis.

The findings presented here rule out the possibility that MDO synthesis occurs via the same lipid carrier mechanism used for synthesis of peptidoglycan, O-antigen, and K1 capsular polysaccharide. However, they do not exclude the use of bactoprenol as a carrier if the biochemistry of MDO release from the carrier differs from the mechanism used for the other oligosaccharides. We consider it unlikely that MDO biosynthesis would be the exception in using bactoprenol as a carrier which is not released as a pyrophosphate derivative following transmembrane transfer of the MDO glycan chain. Rather, the finding of an acyl carrier protein as a soluble factor in MDO biosynthesis [26] points to a translocation mechanism across the cytoplasmic membrane that differs from the transmembrane transfer of the other oligosaccharides.

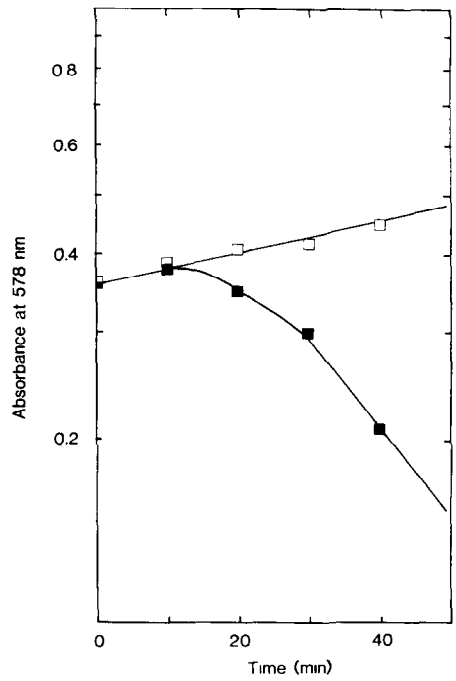

Fig.2. Effect of colicin M treatment on strain EH247 grown in peptone medium. One culture was given colicin M at 0 min (■) while the second culture remained untreated (□).

Table 1

Determination of membrane derived oligosaccharides synthesized by strain EH3247

Time	pmol [^{14}C]glucose incorporated/ml culture	
	Untreated	Colicin M treated
0 min	22.8 (1.0)	20.8 (1.0)
5 min		25.6 (1.2)
10 min	31.3 (1.4)	29.0 (1.4)
20 min	33.8 (1.5)	25.9 (1.2)
30 min	38.8 (1.7)	21.3 (1.0)

Colicin M (0.5 μg protein/ml culture) was added to the treated culture at 0 min. At times indicated, 1 ml samples of the untreated control and colicin M-treated cultures were pulse-labelled with [^{14}C]glucose for 3 min and the amount of radiolabelled glucose incorporated into the MDO fraction was quantitated as described in section 2. Relative values are given in parentheses

Results presented here are an example of how colicin M can be used as a simple tool in determining the involvement of bactoprenol as a transmembrane carrier. Another example where colicin M could be employed as a tool is in the study of enterobacterial common antigen biosynthesis [27]. In general, bactoprenyl-linked intermediates are difficult to identify due to the low concentration of the carrier although huge amounts of precursors (in the order of 6×10^6 molecules in 20 min) are translocated via this lipid across the cytoplasmic membrane.

The paper chromatographic method of isolating and quantitating MDO is both rapid (relative to the more laborious column chromatographic procedures reported in the literature), reproducible, and permits simultaneous separation of many samples with ease.

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