GLYCOSYLATION OF THE SURFACE GLYCOPROTEIN OF *HALOBACTERIUM SALINARIUM* VIA A CYCLIC PATHWAY OF LIPID-LINKED INTERMEDIATES

Matthew F. MESCHER[+] and Jack L. STROMINGER
The Biological Laboratories, Harvard University, Cambridge, MA 02138, USA

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1. Introduction

Assembly of a complex polysaccharide via a cyclic pathway involving polyprenol lipid-linked intermediates was first demonstrated in the case of peptidoglycan biosynthesis [1] and there is now considerable evidence that at least some of the glycoproteins of eukaryotic cells are glycosylated via similar intermediates [2–7]. *Halobacterium salinarium* has a high molecular weight glycoprotein as its major surface component [8,9] and has recently been shown to have enzymatic activities for formation of lipid-linked sugars of the type expected as intermediates if glycosylation of the glycoprotein occurs via a polyprenol lipid carrier [10]. Despite the fact that *Halobacteria* lack a peptidoglycan layer their growth is inhibited by bacitracin [11], an antibiotic which inhibits growth of normal bacteria by complexing with the C_{55} isoprenyl pyrophosphate released upon transfer of the newly formed subunit to the growing peptidoglycan chain [12,13]. Formation of this complex prevents the enzymatic dephosphorylation of the lipid which is necessary for it again to act as an acceptor and allow continued peptidoglycan synthesis. The effects of bacitracin on *H. salinarium* have been examined and this report describes experiments which provide further evidence that glycosylation of the surface glycoprotein occurs via a cyclic pathway involving lipid intermediates.

[+] Present address: Department of Pathology, Harvard Medical School, Boston, MA 02115, USA

2. Materials and methods

Bacitracin was obtained from The Upjohn Co. (Kalamazoo, MI). UDP-*N*-acetyl-[^{14}C]glucosamine (269 mCi/mmol) was obtained from Amersham Searle and L-[^{35}S]methionine and sodium [^{32}P]-phosphate (carrier-free) from New England Nuclear.

Growth, harvesting and cell envelope preparation of *Halobacterium salinarium*, strain 1, were as in [8]. [^{35}S]Methionine-labeling of cells was done in a defined medium [14] originally described [15] with the exception that the methionine concentration was reduced from 2.5–0.25 mM. This concentration was sufficient to allow normal growth and approx. 12% of the radioactivity was incorporated by the cells. Phospholipids were labeled by growing cells in the normal growth medium [8] to which approx. 50 μCi/ml of sodium [^{32}P]phosphate was added.

Carbohydrates were determined by colorimetric assays as in [9]. Protein was determined as in [16]. Thin-layer chromatography of lipids and carotenoids was done on precoated plates of Silica Gel F-254 (Merck). Solvents A and B were chloroform : methanol : water (65:35:6) and (65:25:4) and C was 2,6-dimethyl-4-heptanone : acetic acid:water (8:5:1).

3. Results

When bacitracin is added to growing cultures of *H. salinarium*, cell growth continues for approximately one doubling time and a gradual change from normal rod-shaped to spherical cells occurs [11]. The surface

glycoprotein is the major shape-maintaining component of the cell envelope [17], suggesting that bacitracin may act by altering its synthesis or composition. The proteins of cells grown in the presence of [^{35}S]methionine were examined in order to determine if the glycoprotein continues to be made and if it is present to the same extent in both normal and bacitracin-treated cells.

Labeling was begun 2 h after addition of bacitracin to the treated culture and was continued for 24 h (fig.1). At the end of this time all (> 98%) of the cells grown in the presence of bacitracin were spherical and all of the untreated cells were rod-shaped. Labeled cells were then harvested and washed in [^{35}S]methionine-free medium. The final cell pellet was then solubilized in 2% SDS and the proteins were

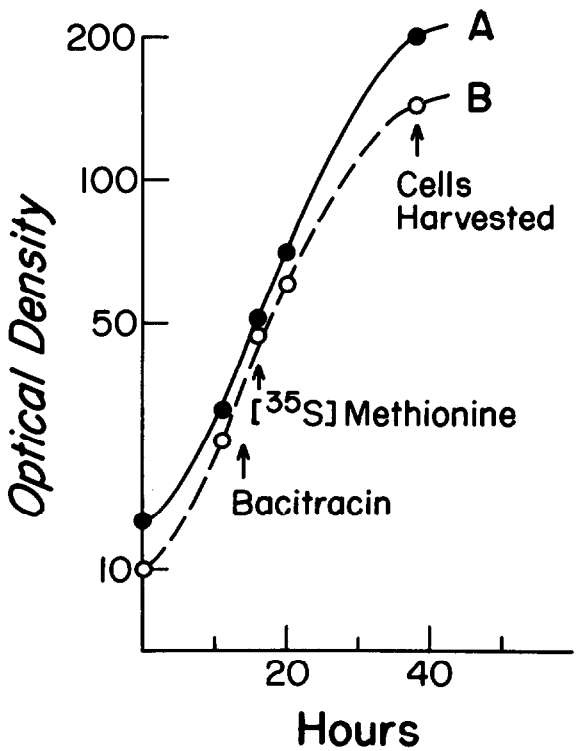

Fig.1. Growth curves and [^{35}S]methionine labeling of untreated (A) and bacitracin-treated (B) cells. Growth was determined using a Klett photometer with a red filter. Bacitracin was added to culture B at final conc. 25 μg/ml. [^{35}S]-Methionine (approx. 18 μCi) was added to both cultures 2 h later. Untreated cells incorporated approx. 12% added label and treated cells approx. 6%.

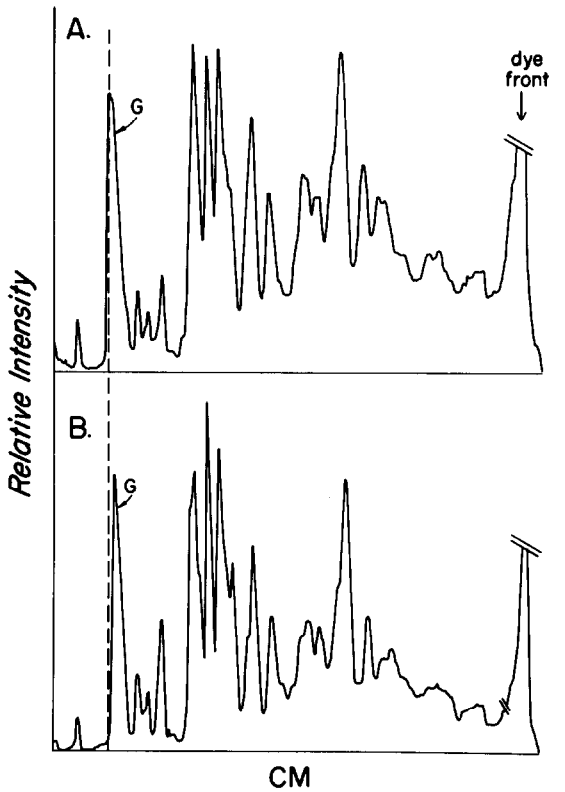

Fig.2. Proteins of [^{35}S]methionine-labeled *H. salinarium*. Proteins of cells grown and labeled as shown in fig.1 were electrophoresed on SDS–polyacrylamide slab gels using the gel system in [18] with a 3% acrylamide stacking gel and a 7.5% acrylamide running gel. Shown are densitometry scans of autoradiograms of dried gels of (A) untreated cells and (B) cells grown in the presence of bacitracin. The glycoprotein is labeled G and the dotted line is included to illustrate the slightly higher mobility of the glycoprotein in treated cells. Quantitation of radioactivity was done by slicing bands from the gels, incubating overnight at 30°C in 10 ml toluene scintillation fluid containing 3% protosol (New England Nuclear) and counting in a liquid scintillation counter. Identical results were obtained when samples were run on tube gels and sliced and counted as in [8].

examined by SDS–polyacrylamide gel electrophoresis (fig.2). The glycoprotein accounted for 11–13% total radioactivity in the proteins of both untreated and bacitracin-treated cells. Thus, while bacitracin inhibited cell growth and caused a morphological change it did not selectively inhibit synthesis of the glycoprotein.

Although the glycoprotein was present to the same

extent in normal and treated cells, its migration during SDS–polyacrylamide gel electrophoresis indicated that it might have an altered composition in treated cells. The glycoprotein of treated cells has a slightly higher mobility (fig.2 and [17]), consistent with it having a decreased carbohydrate content. Purification by aqueous phenol extraction was attempted in order to obtain the altered glycoprotein for further characterization. This procedure results in separation of the normal glycoprotein, which remains in the aqueous phase, from the other cell envelope proteins which are extracted into the phenol phase [8,9]. However, when envelopes from bacitracin-treated cells were extracted, the glycoprotein was found in the phenol phase along with the other envelope proteins. A decreased carbohydrate content would explain the failure of the altered glycoprotein to partition into the aqueous phase.

Due to the fact that the glycoprotein accounts for all of the non-lipid carbohydrate of the cell envelope [8] it has been possible to demonstrate that bacitracin treatment results in decreased glycosylation despite the inability to isolate the altered glycoprotein from treated cells. 25% neutral hexose in the cell envelope is present in (2:1) chloroform:methanol-extractable glycolipids [8]. The other 75% of the hexose and all of the amino sugar is attached to the glycoprotein in the form of a single *N*-linked heterosaccharide containing amino sugars and hexoses and 34–38 *O*-linked di- and tri-saccharides containing neutral hexoses and hexuronic acid [9]. Lipid-free envelopes from bacitracin-treated cells showed significant decreases in all of the carbohydrates in comparison to envelopes of untreated cells (table 1). These results, together with the evidence that the glycoprotein continues to be synthesized and is present to the same extent in normal and treated cells, demonstrate that growth in the presence of bacitracin results in inhibition of glycosylation of the glycoprotein. The greater decrease in amino sugar than in hexose or uronic acid suggests that synthesis and/or attachment of the *N*-linked heterosaccharide is affected to a greater extent than is that of the *O*-linked units.

The other major envelope components have been examined in order to determine if bacitracin affects only the glycoprotein. These include the remaining proteins (approx. 32% envelope dry wt) and the lipids (approx. 21% envelope dry wt) [8]. It was previously shown that no changes in the envelope proteins, other than the glycoprotein, are detectable by SDS–polyacrylamide gel electrophoresis ([16] and fig.2). No significant difference in the total amount of glycolipid was found between untreated and treated cells (table 1). No differences in lipid composition were detected when total lipid extracts ($CHCl_3$: MeOH, 2:1) were compared by thin-layer chromatography on silica gel in solvent systems A, B and C followed by iodine staining. Similarly, no differences in major phospholipids were detected when cells were labeled with ^{32}P and the lipids examined by thin-layer chromatography in solvent systems A, B and C, followed by autoradiography. Two very minor phospholipids (accounting for less

Table 1
The effect of bacitracin on cell-envelope carbohydrate content[a]

	(μmol/100 mg envelope protein)			
	Amino sugar	Hexose	Uronic acid	Glycolipid hexose
Untreated[b]	7.9 ± 0.6	35.6 ± 0.6	17 ± 2	10.3 ± 0.2
Bacitracin[b] treated	4.0 ± 0.6	24 ± 2	10.5 ± 0.3	9.7 ± 0.3
% decrease	49	32	38	6

[a] Isolated cell envelopes were extracted 3 times with $CHCl_3$:MeOH (2:1) and the lipid-free cell envelopes assayed for protein and carbohydrate content as in section 2. Extracted lipids were evaporated to dryness, redissolved in $CHCl_3$:MeOH (2:1) and aliquots assayed

[b] Bacitracin (25 μg/ml) was added to the treated cultures 12 h after inoculation and control and treated cells harvested 24 h later

than 0.5% total radioactivity) were greatly decreased or absent in bacitracin-treated cells. The possible nature of these will be discussed below.

The carotenoids present in the envelope of *H. salinarium* [19] were also examined in treated and untreated cells. In vitro synthesis of compounds whose biosynthetic pathways involve polyprenol pyrophosphate intermediates, as is the case for carotenoids, is inhibited by bacitracin [20,21], presumably due to complex formation between bacitracin and the polyprenol pyrophosphate precursors. No differences in either carotenoid content (measured as *A* units/mg envelope protein) or in the absorption spectra of the carotenoids (fig.3) was found between untreated and bacitracin-treated *H. salinarium*. There were also no differences between treated and untreated cells in the mobilities of the three major carotenoids on silica gel in solvent systems A, B and C. These results indicate that bacitracin is unable to enter the cell and suggest that its action in inhibiting glycosylation of the glycoprotein occurs at the cell surface.

The absence of two minor phospholipids in bacitracin-treated cells might be explained by the inability of these cells to form the lipid intermediates for glycosylation due to all polyprenol being in the pyrophosphate form complexed with bacitracin. This

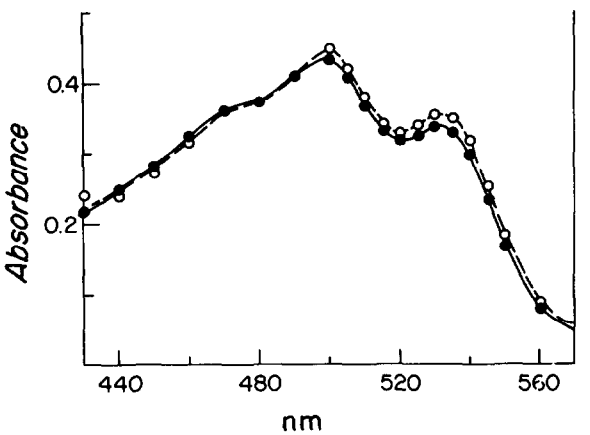

Fig.3. Absorption spectra of carotenouds from (-•-•-) normal and (—o— —o—) bacitracin treated cells. Cells were grown and treated as shown in fig.1. Cell envelopes were extracted with $CHCl_3$:MeOH, (2:1) and the extracts dried and redissolved in $CHCl_3$:MeOH (1:1) at conc. equiv. 0.67 mg cell-envelope protein/ml.

Table 2
Formation of C_{55-60} isoprenyl pyrophospho-*N*-acetylglucosamine by cell homogenates of untreated and bacitracin-treated cells

	pmol amino sugar incorporated/100 mg protein
Untreated	4.7 ± 0.3
Bacitracin-treated[b]	0.8 ± 0.3
Untreated + bacitracin[c]	4.8 ± 0.3

[a] Cell homogenates were prepared and assayed as in [10]. Reaction mixture was final vol. 0.25 ml and UDP-*N*-acetyl-[^{14}C]glucosamine was added at final conc. 1.5 μM

[b] Cells were grown and treated as shown in fig.1 and harvested 20 h after the addition of bacitracin (25 μg/ml) to the culture

[c] Bacitracin was added to the normal cell homogenate reaction mixture at final conc. 25 μg/ml prior to addition of the UDP-*N*-acetyl-[^{14}C]glucosamine

possibility was tested more directly by examining lipid-intermediate formation. It was previously shown that cell homogenates have enzymatic activities for formation of lipid-linked sugar compounds from nucleotide sugars and endogenous lipid. In the case of UDP-*N*-acetylglucosamine, the only product formed is C_{55-60} isoprenyl pyrophospho-*N*-acetylglucosamine. As expected if bacitracin is acting by blocking a cyclic lipid carrier pathway, formation of this compound was greatly reduced (75–90%) in cells grown in the presence of bacitracin (table 2). That transfer of the sugar from the C_{55-60} lipid to an acceptor with release of lipid pyrophosphate does not occur under in vitro assay conditions [10]. Addition of bacitracin to a homogenate of untreated cells should therefore not affect formation of the *N*-acetylglucosamine lipid and the results shown in table 2 confirm this.

4. Discussion

The effects of bacitracin described here, together with the results of studies of the formation of lipid-linked sugar compounds [10], suggest that formation of the *N*-linked heterosaccharide of the *H. salinarium* surface glycoprotein occurs via a cyclic pathway involving lipid-linked intermediates. The heterosaccharide is apparently formed at the cytoplasmic

side of the membrane by sequential transfer of sugars to the lipid carrier from substrates available in the cytoplasm. *N*-Acetylglucosamine is added first to form isoprenyl pyrophospho-*N*-acetylglucosamine. Additional sugars are then attached via glycosidic bonds to the growing saccharide chain by transfer from either nucleotide diphosphosugars or isoprenyl monophospho-sugars [10]. The heterosaccharide is then transferred, at the exterior surface of the cell, to the protein acceptor and the resulting lipid pyrophosphate is dephosphorylated to yield the monophosphate which can again act as an acceptor. Bacitracin blocks the dephosphorylation, presumably by complexing with the lipid pyrophosphate at the outer cell surface, thus inhibiting further glycosylation. There is not sufficient evidence available to allow a conclusion as to whether the *O*-linked di- and tri-saccharides of the protein are formed via a similar pathway. Attachment is via an *O*-glycosidic linkage between galactose and threonine but we have been unable to demonstrate either formation of a galactose–lipid compound [10] or direct transfer of galactose from nucleoside diphosphogalactose to a deglycosylated acceptor protein (unpublished). The decreased amount of *O*-linked carbohydrate on the glycoprotein of cells grown in bacitracin (table 1) suggests that lipid intermediates might be involved but the possibility that this decrease is a secondary effect of inhibiting attachment of the *N*-linked heterosaccharide cannot be ruled out.

While it is clear that glycosylation of eukaryotic glycoproteins can occur via dolichol-linked intermediates [2–7] it has not been demonstrated that this is a major pathway for glycosylation nor that it is cyclic. We have been unsuccessful in attempting to demonstrate an effect of bacitracin on glycosylation of glycoproteins in a human lymphocyte cell line grown in vitro despite the fact that bacitracin can form a complex with dolichol pyrophosphate (P. Parham, M. F. M. and J. Wedgwood, unpublished). As is the case with *Halobacteria*, bacitracin appears to be unable to enter the cell, as evidenced by normal levels of cholesterol biosynthesis. A likely explanation for failure to observe an effect of bacitracin on glycosylation of lymphocyte glycoproteins is that formation and attachment of saccharide units probably occurs primarily on the endoplasmic reticulum or in the Golgi apparatus rather than at the surface of the plasma membrane and that the dolichol pyrophosphate released upon transfer is thus inaccessible to binding by bacitracin.

Acknowledgement

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