Role of the ftsA1p promoter in the resistance of mucoid mutants of Salmonella enterica to mecillinam: characterization of a new type of mucoid mutant

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Accepted 10 May 2001
First published online 5 June 2001

Abstract

Mucoid mutants of Salmonella enterica serovar Typhimurium isolated by resistance to mecillinam include lon (27%) and rcsC (8%) mutants but the most frequent class (65%) is affected in a new gene (mucM) located at centisome 76. mucM cells are shorter than mucM‡ cells and rcsB mutations normalize size and response to mecillinam. Expression of ftsA1p, the ftsA-ftsZ promoter submitted to RcsB stimulation, is greatly increased in mucM mutants, and this expression is dependent on RcsB and ftsA1p. It is proposed that the mucM product interferes with RcsB activation. Mucoidy results from the activation of cps genes and mecillinam resistance from ftsA-ftsZ overexpression, both traits caused by the increased activity of the RcsB effector. The same mechanism seems to be responsible for the resistance of mucoid rcsC mutants to mecillinam but the resistance of lon mutants is not dependent on RcsB and so responds to a different cause. © 2001 Federation of European Microbiological Societies. Published by Elsevier Science B.V. All rights reserved.

Keywords: Mecillinam; Mucoid mutant; FtsZ; Salmonella; Escherichia coli

1. Introduction

Mucoid colonies of Escherichia coli and Salmonella enterica are frequently observed among survivors of selective agents or detrimental conditions, but although several protective properties have been assigned to the production of capsule, conclusive evidences concerning eliciting factors or advantages have not yet been obtained. Regulation of capsule synthesis has been thoroughly studied in E. coli K-12, and evidence for a dual control has been gathered [1]. Thus, synthesis of colanic acid (CA), the capsule exopolysaccharide produced by most Enterobacteria, depends on the expression of a cluster of 19 genes (cps genes) [2,3] which is controlled by a two-component system formed by rcsB and rcsC genes. When activated by the RcsC component, probably through phosphorylation, RcsB acts as a positive effector for cps transcription. RcsC is a large transmembrane protein which is thought to sense and respond to environmental conditions. Another factor affecting capsule synthesis, perhaps by participating in RcsB activation, is the protein encoded by gene rcsF [4]. A second regulatory mechanism depends on the positive action of the product of gene rcsA. RcsA is a short-lived protein which interacts with RcsB to optimize cps transcription. The level of the RcsA protein is limiting for CA synthesis and is kept very low by its sensitivity to the Lon protease. In fact, the most common class of mucoid mutant isolated at 32°C corresponds to lon mutants [5] that produce high amounts of CA because of the increased level of RcsA. Although rcsA mutations drastically decrease transcription of cps genes, only inactivation of rcsB gene completely prevents CA synthesis. Several other factors influence CA production either by modifying the availability of RcsA (hns and dsr mutations) or by evoking RcsC response (dshB and some LPS core mutations) (see Gottesman [1] for a review). In addition to its central role as the regulator of cps transcription, RcsB stimulates synthesis of the essential cell division protein FtsZ [6]. Recently, Carballés et al. [7] demonstrated that the action of RcsB is specifically exerted on the ftsA1p promoter and results in increased expression of both ftsA and ftsZ genes.
The lethal action of mecillinam, a β-lactam antibiotic that specifically inhibits penicillin-binding protein (PBP) 2, the PBP involved in maintenance of bacillus cell shape in Enterobacteria [8], results in the appearance of many classes of resistant colonies among which large mucoid colonies are very conspicuous. The origin of those mucoid derivatives has not been traced but it is assumed that they are a defensive response to the antibiotic’s presence. In this paper, mucoid mutants of S. enterica serovar Typhimurium LT2 were selected by resistance to mecillinam and their nature was investigated. It was found that most of those mutants belong to a new class of mucoid mutants which has been called mucM. The characteristics of these strains suggest that mucM mutations elicit CA synthesis by activating the RcsC/RcsB response. The resistance of mucM mutants to mecillinam would be due to overexpression of ftsZ and/or ftsA genes caused by the increased activity of the RcsB effector.

2. Materials and methods
2.1. Bacterial strains, phage and media

All the strains used were derivatives of S. enterica serovar Typhimurium LT2 unless otherwise specified. Mucoid mutants were isolated by plating independent stationary cultures of DA1468, an argC95 mutant used as the standard strain in this laboratory, on LB agar containing 1 μg ml⁻¹ mecillinam. Mutation mucM5 was obtained by the same procedure in a different strain and then transferred by transduction to DA1468. In the latter strain the mucM5 derivative is only slightly mucoid on LB agar but clearly mucoid on minimal medium. Mucoid mutants were characterized as described in the text. The cps-I::MudJ fusion was isolated by the procedure of Hughes and Roth [9] by looking for red non-mucoid colonies among kanamycin-resistant transductants of strain DA1930 (rcsC11) selected on MacConkey-kanamycin agar. The cps-I::MudJ mutation is linked by transduction to the rfb cluster (40%) but not to the histidine operon. Tn10d(Tet) insertions in rcsA, rcsB and cps genes were obtained by transducing strain DA2026 (mucM1 cps-I::MudJ) with a lysate grown in a pool of random Tn10d(Tet) insertions. Tetracycline-resistant transductants that produced white colonies on MacConkey-tetracycline agar where characterized by transduction. Cotransduction with the serU locus (20%) served to identify rcsA mutants, and cotransduction with the gyrA gene (40%) to identify rcsB strains.

Transductions were mediated by phage P22 HT105/1 int-201. All the strains constructed by transduction were obtained free of phage by successive single-colony isolations.

Complete medium was LB broth and LB agar, and E medium was used as minimal medium [10]. MacConkey agar was used to test fermentation of lactose. Antibiotics were used at the following concentrations: mecillinam, 1 μg ml⁻¹; kanamycin, 20 μg ml⁻¹; tetracycline, 20 μg ml⁻¹; nalidixic acid, 20 μg ml⁻¹. Mecillinam was a kind gift of Leo Pharmaceutical Products (Denmark).

2.2. β-Galactosidase assay and other methods

β-Galactosidase activity of cps-I::MudJ, pFAB4 and pFAB3 strains was assayed in stationary cultures grown in LB broth containing 20 μg ml⁻¹ kanamycin at 37°C. Exponential cultures of pFAB4 and pFAB3 strains grown as described by Carballès et al. [7], but with IPTG omitted, were also assayed. The assay was performed as described by Miller [11] on cells treated with sodium dodecyl sulfate and chloroform. Specific activities are expressed in Miller units referred to OD₆₅₀.

MIC of mecillinam was determined by plating cell from stationary cultures on LB plates containing twofold serial dilutions of the antibiotic [12].

3. Results and discussion
3.1. Isolation of mucoid mutants by resistance to mecillinam

Mecillinam-resistant colonies appear at a frequency of about 2×10⁻⁶ when stationary cultures of S. enterica serovar Typhimurium are plated on LB agar containing 1 μg ml⁻¹ mecillinam at 37°C. Approximately 5–10% of those resistant colonies display a mucoid phenotype. From six different derivatives of strain LT2, 26 independent mucoid mutants were isolated from mecillinam plates and submitted to a genetic screening to identify the affected gene. To that purpose, Tn10 insertions placed close to genes concerned with CA regulation (lon, rcsC, rcsA) were used [13]. It was found that 7 (27%) of the mucoid isolates carried mutations that cotransduced 50% with marker zaj-1034::Tn10 and showed UV-sensitivity, so they were classified as lon mutants [14]. The mucoid mutations of two other isolates (8%) cotransduced 80% with ompC396::Tn10 and zef-754::Tn10 markers and were classified as rcsC mutants. Mucoidy of the other 17 mutants (65%) failed to show cotransduction with those insertions as well as with fli-8017::Tn10 and zeb-609::Tn10 insertions which are linked to the rcsA gene. The mutation conferring the mucoid phenotype to one of those strains (DA2025) was roughly mapped by isolating a Tn10d(Tet) insertion (zhg-7131::Tn10d(Tet)) placed very close to the gene concerned. The insertion was mapped by using the procedure of Benson and Goldman [15] and the results demonstrated that it was located at about 70–80 centi-somes. Cotransduction tests to markers in that zone shown that the mutation responsible for mucoidy was cotransducible with markers cysG (26%), aroB (67%), zgh-7131::Tn10d(Tet) (89%), and envZ (46%). Three factor crosses showed the order to be cysG-aroB-zgh-
that extensive screening for mucoid mutants without mecillinam mutants were found with cephalexin. Thus, although an lon mutants were obtained with cefsulodin and only two alexin (highly specific for PBP 3) were used. No mucoid L than mecillinam also selected mucM mutations are recessive to mucM inner membrane, because, like wild-type bacteria, mucM cells became spherical when placed in LB broth containing 1 μg ml⁻¹ mecillinam. In order to test if β-lactams other than mecillinam also selected mucM mutants, cefsulodin (a β-lactam antibiotic specific for PBP 1A and 1B) and cephalaxin (highly specific for PBP 3) were used. No mucoid mutants were obtained with cefsulodin and only two lon mutants were found with cephalaxin. Thus, although an extensive screening for mucoidy and resistance to mecillinam was observed in the transductions performed; therefore, the two characteristics appear to be caused by the same mutation. Resistance to mecillinam was not due to failure of the antibiotic to reach PBP 2, its target in the inner membrane, because, like wild-type bacteria, mecillinam was observed in the absence of Lon protease strikingly increased the mutation was tested, the high level of RcsA caused by the rcsC11 derivative. Phase contrast photomicrography of wild-type (a), mucM1 rcsB derivative. Fig. 1. Cell morphology of mutant mucM1 and its rcsB17 derivative. Phase contrast photomicrography of wild-type (a), mucM1 rcsB17 (b) and mucM1 rcsB17::Tn10d(Tet) (c) strains grown to late exponential phase in minimal medium.

3.2. Characteristics of mucM mutants

The effect of mutations in genes concerned with capsule regulation on the behavior of mucM mutants was investigated. As shown in Table 1, rcsB mutations prevented CA synthesis in mucM mutants. On the other hand, although a strong decrease in cps expression was observed in rcsA derivatives of mucM strains, a low level of β-galactosidase synthesis was maintained (Table 1). Attempts to introduce a mucoid rcsC allele (rcsC11) into mucM mutants failed in every case but when the recipient strain carried mucM5, a very weak mucM allele that causes mucoidy and resistance to mecillinam on minimal medium but not on LB agar. In this case, cps expression in the mucM5 rcsC11 strain increased to 383 units. When a lon mutation was tested, the high level of RcsA caused by the absence of Lon protease strikingly increased β-galactosidase synthesis in mucM strains (Table 1). The response of those double mutants to mecillinam was assayed. It was observed that all of them maintained resistance to the CA, per se, was not required for resistance. The same result was obtained when CA synthesis was prevented by introduction of a mutation (mucA51::MudJ) that stopped the supply of fucose, one of the components of CA.

Table 1

<table>
<thead>
<tr>
<th>Strain</th>
<th>β-Galactosidase activity and MIC of mecillinam on derivatives</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>rcsA16</td>
</tr>
<tr>
<td>wild-type</td>
<td>2.1 (0.08)</td>
</tr>
<tr>
<td>mucM1</td>
<td>424 (20)</td>
</tr>
<tr>
<td>mucM5</td>
<td>7.6 (0.08)</td>
</tr>
<tr>
<td>mucM8</td>
<td>894 (80)</td>
</tr>
</tbody>
</table>

All the strains carried fusion cps-1::MudJ. The regulatory mutations were rcsA16::Tn10d(Tet), rcsB17::Tn10d(Tet) and lon-56::Tn10d(Tet). β-Galactosidase activity is expressed in Miller units referred to OD₆₉₀ [11] and MIC (in parentheses) in μg ml⁻¹. MIC results are representative results of at least three independent experiments. All the assays of β-galactosidase activity were performed in duplicate and the results are average of at least two independent experiments.
antibiotic with the exception of rcsB derivatives that recovered normal sensitivity (Table 1). The response to mecillinam of rcsB derivatives of mucoid rcsC11 and lon-54 mutants (both mutants displaying a MIC of mecillinam of 20 \( \mu \)g ml\(^{-1} \)) was also tested. While the rcsC11 rcsB strain recovered the sensitivity of the wild-type, the lon-54 rcsB derivative maintained the same resistance as the lon-54 rcsB\(^{+} \) strain. A high frequency of non-mucoid colonies was observed in mucM stocks upon subculture or even after short storage. It was found that those colonies still carried the mucM mutation and most of them due to their non-mucoid genotype to the appearance of rcsB mutations while the rest were non-mucoid because of rcsA mutations. All the spontaneous rcsB derivatives recovered sensitivity to mecillinam whereas rcsA derivatives retained resistance to that antibiotic.

3.3. Effect of mucoid mutations on the expression of \( ftsA \) gene

It was observed that cultures of mucM mutants presented a high frequency of short cells at the end of exponential phase, and cell size returned to normal in rcsB derivatives (Fig. 1). The cell size alteration displayed by these mutants could be due to increased activity of the cell division protein FtsZ [17] since it has been demonstrated that RcsB stimulates transcription of \( ftsZ \) and \( ftsA \) genes by acting on the \( ftsA1p \) promoter [7]. Furthermore, overproduction of the proteins encoded by genes \( ftsQAZ \) has been reported to confer mecillinam resistance [18]. In order to test if the resistance of mucoid mutants to mecillinam was related to overproduction of FtsZ, pFAB4 and pFAB3 plasmids, kindly provided by K. Cam, were used. Plasmid pFAB4 carries a lacZ reporter gene fused to a 242 bp fragment of the \( ddl-ftsQ-ftsA-ftsZ \) promoter region comprising \( ftsA1p \), the ftsZ promoter submitted to RcsB stimulation. Plasmid pFAB3 contains the same region but with \( ftsA1p \) deleted.

Derivatives of mucM, rcsC, and lon strains carrying either pFAB4 or pFAB3 plasmids were prepared and assayed for \( \beta \)-galactosidase activity. The results are presented in Table 2. It can be seen that mucM mutations increased the \( \beta \)-galactosidase activity from plasmid pFAB4 strongly both at exponential and stationary phase. The results with rcsC11 mutant were similar. In contrast, the lon mutant showed a modest increase in exponential phase and almost the same level as the wild-type at stationary phase. In all the cases the effect was dependent on the presence of the \( ftsA1p \) promoter since it was not observed when the strains carried the pFAB3 plasmid (Table 2).

The effect of rcsB and rcsA mutations on the stimulatory action of mucM mutations was tested. As shown in Table 3, rcsB mutations returned the level of expression of pFAB4 in a mucM mutant to normal whereas rcsA mutations did not alter the increased \( \beta \)-galactosidase activity of the strain. These results demonstrate that resistance of mucM mutants to mecillinam is related to a high level of

### Table 2

| Strain | \( \beta \)-Galactosidase activity of derivative carrying: | | | | |
|---|---|---|---|
| | pFAB4 | pFAB3 | | |
| | exponential phase | stationary phase | exponential phase | stationary phase | |
| wild-type | 104 | 1298 | 7.4 | 42 | |
| mucM1 | 1462 | 9680 | 6.1 | 35 | |
| mucM8 | 5013 | 25713 | 10 | 32 | |
| rcsC11 | 571 | 9662 | 8 | 41 | |
| lon-54 | 289 | 1786 | 14 | 45 | |

Plasmid pFAB4 carries a lacZ fusion to the \( ftsA1p \) promoter; pFAB3 carries the same fusion but with \( ftsA1p \) deleted. Mucoid mutants rcsC11 and lon-54 were isolated by resistance to mecillinam. Conditions as described in Section 2 and Table 1.

### Table 3

| Strain | MIC (\( \mu \)g ml\(^{-1} \)) | \( \beta \)-Galactosidase activity of derivative carrying: | |
|---|---|---|
| | | \( ftsA1p-lacZ \) | | | |
| | | exponential phase | stationary phase | | |
| wild-type | 0.08 | 2.1 | 104 | 1298 | |
| rcsA16 | 0.08 | 2.0 | 115 | 1238 | |
| rcsB17 | 0.08 | 2.0 | 108 | 1172 | |
| mucM1 | 40 | 424 | 1462 | 9680 | |
| mucM1 rcsA16 | 40 | 11 | 1067 | 7442 | |
| mucM1 rcsB17 | 0.08 | 2.4 | 104 | 1156 | |

MIC of mecillinam on \( cps^{+} \) derivatives of the corresponding strains. The regulatory mutations were rcsA16::\( Tn10d \) (Tet) and rcsB17::\( Tn10d \) (Tet). Conditions as described in Section 2 and Table 1.
ftsA1p activity but not to the CA level. They also confirm previous reports that the RcsA protein is not involved in stimulation of ftsZ expression by RcsB [4]. Moreover, the data in Table 3 indicate that, at least under the conditions used, the basal level of ftsA1p expression was not mediated by RcsB because the rcsB mutant presented the same level of β-galactosidase activity as the wild-type.

There is no simple explanation for the striking abundance of mucM mutants among mucoid mecillinam-resistant strains or for their absence among unselected mucoid mutants. The mucoid phenotype of mucM mutants is not dependent on the presence of mecillinam, and mucM mutations are stable since their spontaneous non-mucoid derivatives are not true reversions but caused by rcsB or rcsA suppressors. It is noteworthy that although mucoid rcsC mutants are very similar to mucM mutants, rcsC mutants are frequently isolated without help of mecillinam. On the other hand, the resistance displayed by lon mutants to mecillinam would have a different origin from that of mucM and rcsC mutants because they display an almost normal level of ftsA1p activity and their rcsB derivatives maintain resistance to mecillinam.

Results reported by Navarro et al. [18] indicate that all three cell division proteins FtsQ, FtsA and FtsZ must be overproduced for mecillinam resistance to appear in E. coli K-12. The present work is concerned with ftsA1p, which is a minor promoter among those controlling expression of the ftsQAZ cluster [19] and is limited to the control of only ftsZ and ftsA genes [7]. Although overexpression of FtsA and/or FtsZ could perhaps be enough to confer mucillinam resistance to S. enterica, the possibility that RcsB also affects the FtsQ level or modifies other functions leading to resistance is not dismissed by the results reported here.

The role of the mucM product in the regulation of CA synthesis is open to speculation. Although it seems to act as a negative regulator of cps expression, preliminary results indicate that mucoidy of mucM strains depends on RcsC. Therefore, MucM could influence the sensor activity of RcsC either by altering the conditions sensed by RcsC, as dsbB and certain LPS mutations are supposed to do, or directly by affecting RcsC interaction with RcsB.

Acknowledgements

Thanks are due to K. Cam for pFAB4 and pFAB3 plasmids, and to K.E. Sanderson and J.R. Roth for many strains provided. The excellent technical assistance of Ana Maria Flores is gratefully acknowledged. This work was supported in part by a grant of Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET) of Argentina. D.N.A. is a Career Investigator of CONICET.

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