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Occurrence of the Regulatory Nucleotides ppGpp and pppGpp following Induction of the Stringent Response in Staphylococci

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The stringent response in *Escherichia coli* and many other organisms is regulated by the nucleotides ppGpp and pppGpp. We show here for the first time that at least six staphylococcal species also synthesize ppGpp and pppGpp upon induction of the stringent response by mupirocin. Spots corresponding to ppGpp and pppGpp on thin-layer chromatograms suggest that pppGpp is the principal regulatory nucleotide synthesized by staphylococci in response to mupirocin, rather than ppGpp as in *E. coli*.

Bacteria adapt to amino acid or carbon source insufficiency by a complex series of regulatory events known as the stringent response (reviewed in reference 4). In *Escherichia coli*, for example, amino acid starvation leads to an accumulation of uncharged cognate tRNA. When the ratio of charged to uncharged tRNA falls below a critical threshold (24), occupation of the vacant mRNA codon at the ribosomal A site by uncharged cognate tRNA leads to stalling of peptide chain elongation and synthesis of the nucleotides pppGpp and ppGpp from GTP and ATP in an idling reaction involving RelA (2, 9, 13). Intracellular concentrations of (p)ppGpp are controlled by the *relA* gene, encoding the ribosome-dependent (p)ppGpp synthetase I, or stringent factor (19), and the *spoT* gene, encoding the ribosome-independent ppGpp 3′-pyrophosphohydrolase/ppGpp synthetase II (14, 33).

Induction of the stringent response rapidly reduces the synthesis of mRNA because of inhibition of RNA polymerase by ppGpp (28, 31). This coincides with the down-regulation of a wide range of energetically demanding cellular processes, including the synthesis of stable RNA, DNA, protein, and peptidoglycan. Close coordination between transcription and translation can be maintained through the effects of the stringent response on the regulation of individual ribosomal proteins; elongation factors G, Tu, and Ts; certain aminoacyl tRNA synthetases; and stationary-phase-specific sigma factor (σ^s) (11). Ultimately, changes such as these enhance survival in a nutrient-poor environment.

The antibiotic mupirocin (30) produces cellular effects similar to those of isoleucine starvation by preventing the charging of $tRNA^{Ile}$ due to inhibition of isoleucyl tRNA synthetase in *E. coli* (16, 17), *Staphylococcus aureus* (5), and other organisms, thereby inhibiting protein synthesis. In the presence of mupirocin, RNA synthesis in *E. coli* and *S. aureus* is also inhibited as a consequence of the stringent response; however, this inhibition can be reversed by chloramphenicol (15), which in *E. coli*, binds close to the binding site for the terminal CCA of aminoacyl tRNA in the peptidyl transferase A site (23) and reduces the amount of RelA-dependent (p)ppGpp synthesis (8, 10). A stringent response mediated by (p)ppGpp has been detected in some gram-positive and gram-negative bacteria and higher organisms after amino acid starvation or induction by a variety of antibiotics (7, 18, 26). However, in halobacteria (25) and streptococci (21), stringency is not necessarily coupled with (p)ppGpp production. Thus, in *Streptococcus pyogenes* and *Streptococcus rattus*, chloramphenicol reverses the inhibition of RNA synthesis caused by mupirocin, but there is no generation of ppGpp in the presence of mupirocin alone. The presence of a different stringent response effector in these streptococci has therefore been postulated.

So far, evidence for (p)ppGpp synthesis in staphylococci has not been reported. Here, we demonstrate induction of (p)ppGpp synthesis in staphylococci treated with mupirocin and confirm that this is linked to behavior typical of the stringent response observed in *E. coli* and other organisms. Finally, we demonstrate the ubiquity of (p)ppGpp generation in a range of clinically relevant staphylococci.

Confirmation of an antibiotic-induced stringent response in *S. aureus* 8325-4. To confirm the existence of a stringent response in *S. aureus* 8325-4, the effect of chloramphenicol on the inhibition of RNA synthesis induced by mupirocin was investigated. RNA synthesis in *S. aureus* 8325-4 (10^7 CFU/ml, grown in brain heart infusion [Oxoid]) was measured by monitoring the incorporation of [5,6-^{3}H]uridine (Amersham [34 Ci/mol; final, 1 μCi/ml]) into trichloroacetic acid precipitates (6) after 40 min of incubation with 500 μg of chloramphenicol (>100× MIC), mupirocin (1×, 2×, or 4× MIC [0.06 μg/ml]), or both per ml. The radiolabelled uridine was equilibrated with the bacteria for 3 min before antibiotics were added. Percentage inhibition values were calculated with respect to controls with and without chloramphenicol.

Chloramphenicol partially reversed the inhibition of RNA synthesis in *S. aureus* 8325-4 caused by mupirocin (Fig. 1a), as found for *S. aureus* Oxford (15) and *E. coli* (Fig. 1b) (8), confirming that *S. aureus* 8325-4 is capable of mounting a stringent response. To investigate the possible role of (p)ppGpp in the stringent response in staphylococci, bacteria were challenged with mupirocin, and the production of these nucleotides was detected as described below.

Detection of (p)ppGpp in mupirocin-treated staphylococci. The method used to detect guanosine polyphosphates was similar to a published procedure (1) with slight modifications. Staphylococcal strains, growing in low-phosphate MOPS (morpholinepropanesulfonic acid) medium (22) supplemented with 2 mg of all amino acids per liter, 10 g of glucose per liter, and

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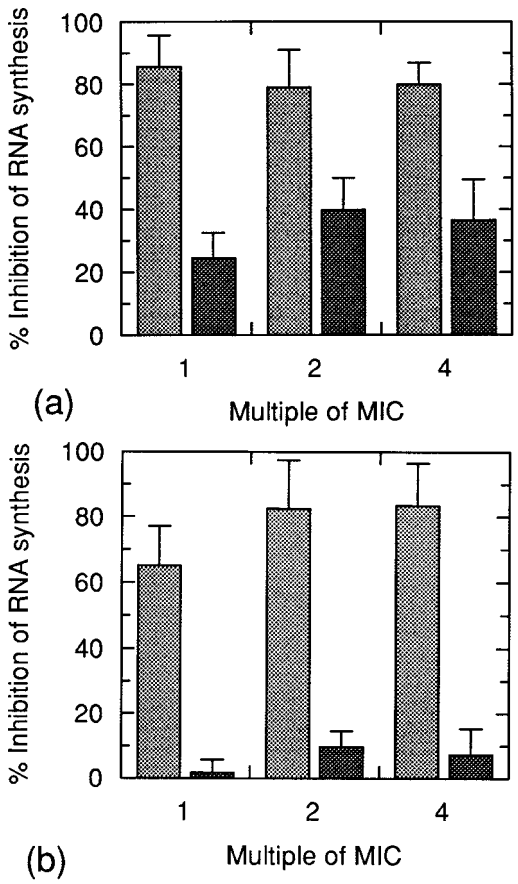

FIG. 1. Induction of the stringent response by mupirocin in *S. aureus* 8325-4 (a) compared with that induced in *E. coli* MG1655 (b). RNA synthesis was measured by monitoring incorporation of [^{3}H]uridine into trichloroacetic acid precipitates as described in the text. The stringent response was induced by addition of mupirocin (1×, 2×, or 4× MIC); reversal of the stringent response was achieved by simultaneous addition of chloramphenicol (500 μg/ml). Inhibition of RNA synthesis was calculated with respect to mupirocin-free controls incubated with (darkly shaded bars) and without (lightly shaded bars) chloramphenicol (n = 4).

10% brain heart infusion, were treated at an optical density at 600 nm (OD_{600}) of 0.1 with $^{33}P_i$ (Amersham [stock of 3,000 Ci/mol; final concentration, 50 μCi/ml of cells]). At an OD_{600} of 0.2 (early exponential phase), cells were treated with mupirocin (60 μg/ml [1,000× MIC]). Samples of the culture (180 μl) were transferred to formic acid (20 μl [10 M]) at various times and frozen on solid carbon dioxide until needed for analysis. After being thawed and incubated on ice for 30 min, samples were spun for 1 min in a microcentrifuge (10,000 × g), and 6-μl aliquots of the clarified supernatants were applied to polyethyleneimine-cellulose thin-layer chromatography (TLC) plates (Merck). The plates were soaked in methanol for 5 min, dried, and then developed in a single dimension by ascending chromatography with 1.2 M KH_2PO_4 (pH 3.5) as an eluant. Nonlabelled standards of GTP, ATP, and ppGpp (ICN Biochemicals) applied to the same plates (and revealed by UV light) eluted as described elsewhere (8). Identification of pppGpp was achieved by comparison of its R_f with published values (2, 8). Treatment of formic acid extracts with activated charcoal (Norit [Sigma]) resulted in the complete disappearance of the spots corresponding to (p)ppGpp, ruling out the presence of nonnucleotide phosphates (10). The presence of polyphosphorylated nucleotides with single substitution on the ribose ring, e.g., (p)pppG or 5′,5′-GpppppG, was ruled out by two-dimensional TLC with 3.3 M ammonium formate–4.2% boric acid (pH 7) in the first dimension and 1.2 M KH_2PO_4 in the second dimension (3). Radiolabelled (p)ppGpp and other phosphorylated species were detected by PhosphorImager analysis (Molecular Dynamics ImageQuant software). Spot intensities for (p)ppGpp were quantified by comparison with that for GTP before addition of mupirocin (1). The combined spot densities for ATP, GTP, and (p)ppGpp did not change significantly during the experiments, suggesting that the increased densities for ppGpp and pppGpp were due to increased synthesis of these nucleotides, probably from GTP and ATP, rather than increased incorporation of ^{33}P.

Treatment of *S. aureus* 8325-4 with mupirocin resulted in the generation of both pppGpp and ppGpp (Fig. 2), their appearance correlating with a reduction in GTP. The spot densities of (p)ppGpp produced in response to treatment by mupirocin

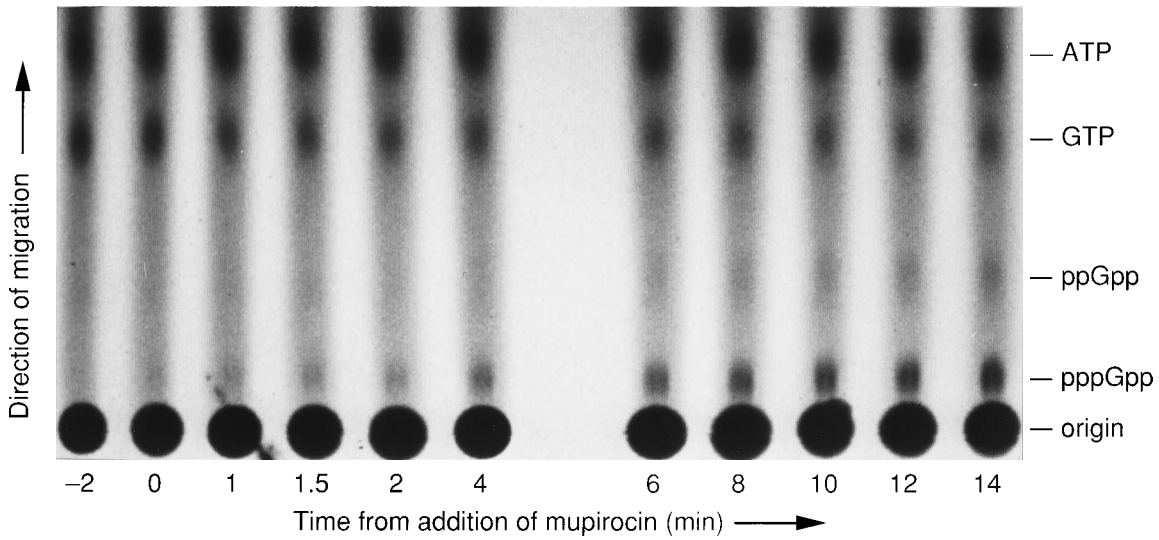

FIG. 2. Generation of ppGpp and pppGpp in *S. aureus* 8325-4 by mupirocin. Bacteria in low-phosphate minimal medium were labelled with $^{33}P_i$ and treated subsequently with mupirocin (60 μg/ml [1,000× MIC]). Formic acid-treated extracts removed at the times shown were subjected to TLC, and the resulting chromatograms were visualized by PhosphorImager analysis. The positions of standards are shown.

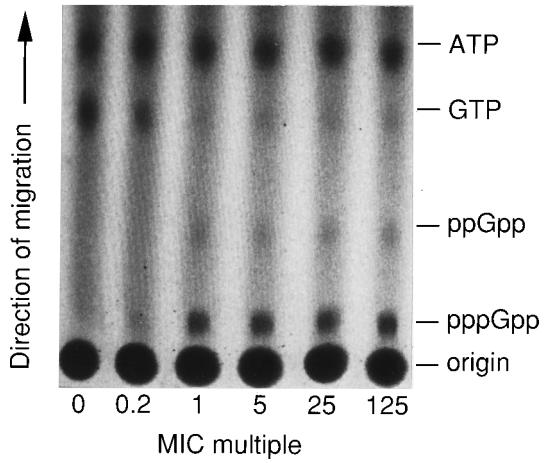

FIG. 3. Generation of ppGpp and pppGpp in *S. aureus* 8325-4. Bacteria in low-phosphate minimal medium were labelled with $^{33}P_i$ and treated subsequently with mupirocin at increasing multiples of the MIC (0.06 μg/ml) as shown. Samples removed 20 min after addition of mupirocin were treated with formic acid, subjected to TLC, and visualized by PhosphorImager analysis.

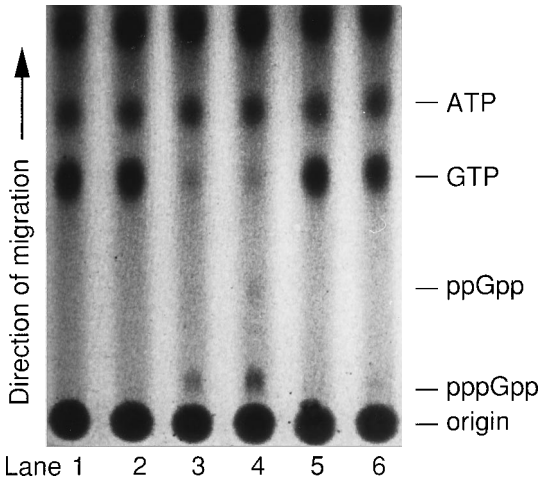

FIG. 5. Association of ppGpp and pppGpp with the stringent response in *S. aureus* 8325-4. The stringent response was induced by mupirocin (0.3 μg/ml [5× MIC]) in ^{33}P-labelled bacteria and relaxed by chloramphenicol (500 μg/ml [>100× MIC]). Samples were removed 20 and 40 min after addition of antibiotics, and formic acid-treated extracts were analyzed by TLC. Lanes: 1 and 2, chloramphenicol; 3 and 4, mupirocin; 5 and 6, chloramphenicol plus mupirocin.

increased as a function of the drug concentration, with detectable amounts even below the MIC (Fig. 3). The generation of (p)ppGpp may therefore be a specific response to inhibition of isoleucyl tRNA synthetase by mupirocin in this experiment, rather than a general response to cellular stress.

In *S. aureus* 8325-4, the densities of the TLC spots corresponding to pppGpp and ppGpp suggested that pppGpp was produced in larger quantities (4.8-fold) than ppGpp, unlike in *E. coli* (0.4-fold). This inversion of the ratio of pppGpp and ppGpp between the two species is shown clearly in Fig. 4. Assuming that pppGpp and ppGpp were labelled to the same extent in each of the two species, this implies that pppGpp rather than ppGpp is the principal nucleotide alarmone in *S. aureus* 8325-4 under these conditions.

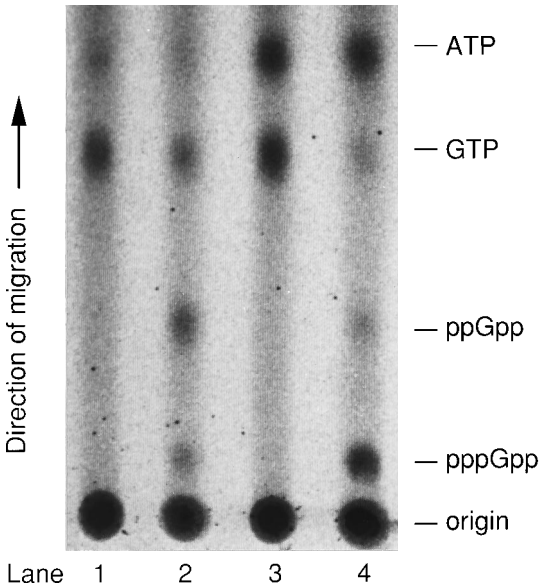

FIG. 4. Relative amounts of ppGpp and pppGpp produced in response to mupirocin in *S. aureus* 8325-4 and *E. coli* MG1655. Bacteria in low-phosphate minimal medium were labelled with $^{33}P_i$ and treated subsequently with mupirocin (1,000× MIC). Samples removed 20 min after addition of mupirocin were treated with formic acid, subjected to TLC, and visualized by PhosphorImager analysis. Lanes: 1 and 2, *E. coli* in the absence (lane 1) and presence (lane 2) of mupirocin; 3 and 4, *S. aureus* in the absence (lane 3) and presence (lane 4) of mupirocin.

Control cultures that were not treated with mupirocin showed no burst of (p)ppGpp synthesis; baseline levels of (p)ppGpp were not detected by this method.

Induction of (p)ppGpp synthesis by other antibiotics. To determine whether (p)ppGpp generation was specific to inhibition of isoleucyl tRNA synthetase by mupirocin, *S. aureus* 8325-4 and *S. aureus* Carter 7 (a clinical strain highly resistant to mupirocin [12]) were treated with indolmycin (an inhibitor of tryptophanyl tRNA synthetase but not isoleucyl tRNA synthetase [32]) at 10× MIC. Accumulation of (p)ppGpp was indeed detected in both of these strains. Treatment of *S. aureus* 8325-4 with a variety of antibiotics other than tRNA synthetase inhibitors did not induce (p)ppGpp generation in every case (data not shown), again supporting the conclusion that this is a specific response to certain antibiotics and not a general response to stress.

The role of (p)ppGpp in mediation of the staphylococcal stringent response. Chloramphenicol reverses the stringent response in wild-type *E. coli*, and this effect is seen as the restoration of RNA synthesis in cells treated with mupirocin (Fig. 1). To examine the effect of chloramphenicol on the generation of (p)ppGpp in mupirocin-treated cells, *S. aureus* 8325-4 was labelled with $^{33}P_i$ (at an OD_{600} of 0.1) as described above. Chloramphenicol (500 μg/ml [>100× MIC]) and/or mupirocin (0.3 μg/ml [5× MIC]) was subsequently added to a split culture at an OD_{600} of 0.2. Aliquots were removed and treated with formic acid as described above after a further 20 and 40 min. Chloramphenicol prevented the generation of (p)ppGpp induced by mupirocin (Fig. 5). These results are consistent with the synthesis of (p)ppGpp in *S. aureus* 8325-4 by a ribosome-dependent process possibly similar to that in *E. coli* (16) and support the involvement of (p)ppGpp in the stringent response in this staphylococcal strain.

How widespread is the ability to synthesize (p)ppGpp among staphylococci? Although the data presented above establish the relationship between (p)ppGpp induction and stringent control in *S. aureus* Oxford and 8325-4, it was important to see

whether the ability to synthesize these nucleotides was a general finding among staphylococci. Thus, a total of 28 strains from six species of staphylococci (*S. hominis* [1 strain], *S. haemolyticus* [2 strains], *S. saprophyticus* [2 strains], *S. warneri* [1 strain], *S. aureus* [15 strains], and *S. epidermidis* [7 strains]) were assayed as described above for (p)ppGpp production after treatment with mupirocin at 100× MIC. All 28 strains synthesized (p)ppGpp, and in every case, pppGpp predominated over ppGpp, in contrast to the situation in *E. coli*. The production of (p)ppGpp therefore appears to be a general response among the staphylococci to treatment with mupirocin.

Conclusions. The data presented here show that staphylococci mount a stringent response, characterized by the generation of (p)ppGpp, when treated with mupirocin to mimic conditions of isoleucine starvation. The occurrence of (p)ppGpp is widespread among the staphylococci, since (p)ppGpp was synthesized in all 28 strains examined (including several of clinical importance bearing methicillin resistance). Generation of (p)ppGpp also occurs with indolmycin, which produces conditions equivalent to tryptophan starvation. We have not examined the generation of (p)ppGpp in staphylococci under conditions of actual amino acid (or carbon source) starvation, but in view of the findings with mupirocin and indolmycin presented above, it is likely to be a response to starvation for amino acids in general (29). Synthesis of (p)ppGpp is accompanied by a fall in intracellular GTP, which is consistent with synthesis from the GTP pool, as has been found in *E. coli*. While the stringent responses appear to be similar in staphylococci, *E. coli*, and various other organisms, one major difference is apparent: the ratio of pppGpp and ppGpp produced in staphylococci is reversed compared with that in *E. coli*, with pppGpp being produced at higher concentrations than ppGpp. While it is too early to speculate on the significance of this for intracellular regulation, it may simply reflect a relatively lower efficiency of the enzyme that converts pppGpp to ppGpp in staphylococci. In *E. coli*, this function is performed by pppGpp 5′-phosphohydrolase (27). Alternatively, the ratio may depend on the position of the culture on the growth curve.

The burst of (p)ppGpp formation in *S. aureus* upon treatment with mupirocin was quenched in the presence of chloramphenicol; this correlates with the ability of chloramphenicol to prevent mupirocin-induced inhibition of RNA synthesis and implies that the stringent response in staphylococci is regulated by (p)ppGpp. Our demonstration that chloramphenicol quenches stringency in both *S. aureus* and *E. coli* confirms previous results (15, 16). Recent studies show that chloramphenicol binds to the ribosomal A site in *E. coli* (23). However, the precise mechanism by which chloramphenicol prevents (p)ppGpp synthesis is not clear, and studies of the effects of chloramphenicol on temperature-sensitive valyl tRNA synthetase mutants under different conditions draw conflicting conclusions (8, 20). This may therefore indicate possible differences between the effective amino acid starvation produced in such mutants and the inhibition of the isoleucyl enzyme by mupirocin. The lower degree of reversal of stringency by chloramphenicol in *S. aureus* compared with that in *E. coli* (Fig. 1) may suggest that the role of uncharged tRNA in RelA-dependent (p)ppGpp synthesis differs in *E. coli* and *S. aureus*. Alternatively, this may be due to inhibition of uridine uptake in *S. aureus* by (p)ppGpp, known to occur in *E. coli* (4). Further examination of this and other properties of (p)ppGpp in staphylococci is therefore warranted.

Identification of the genes that encode (p)ppGpp synthesis and degradation in staphylococci would further our understanding of stringent control in this genus and to what extent it differs from that in *E. coli.*

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