International Journal of Antimicrobial Agents 2 (1993) 263–270

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ANTAGE 00058

Effects of low, subinhibitory concentrations of antibiotics on expression of a virulence gene cluster of pathogenic *Escherichia coli* by using a wild-type gene fusion

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(Accepted 3 February 1993)

S fimbrial adhesins (Sfa) represent virulence factors of E. coli wild-type strains causing urinary tract infections and meningitis of the new-born. In order to determine the influence of subinhibitory concentration of antibiotics on the expression of the sfa gene cluster, a wild-type strain carrying the lacZ gene coding for the enzyme β-galactosidase fused to the sfa determinant was used. The expression of lacZ, which was under the control of the sfa wild-type promoters, was now equivalent to the sfa gene expression of wild-type strain 536. With this strain the influence of subinhibitory concentrations of 28 antibiotics on the expression of the sfa determinant was studied. The expression was strongly suppressed by a treatment of the wild-type fusion strain by aztreonam, gentamicin, clindamycin and trimethoprim; the latter had a dramatic effect on sfa expression. It was further shown for clindamycin and trimethoprim that the reduction of sfa gene expression was dependent on the concentration of the antibiotics. In contrast, imipenem, amphotericin B and rifampicin weakly stimulated sfa expression. We conclude that gene fusions between virulence-associated loci and indicator genes in wild-type pathogens are useful to study virulence modulation due to subinhibitory concentration of antibiotics on the genetic level.

Key words: *E. coli*; S fimbrial adhesin; gene fusion; subinhibitory antibiotic concentration

Introduction

Concentrations of antimicrobial agents below the minimal inhibitory concentration (MIC) have various effects on the bacterial cell which consequently interfere with processes of host-parasite interactions like phagocytosis [1,2], serum resistance of bacteria [3,4] or adherence [5–7]. Such low doses also influence the production of pathogenicity factors but there is no evidence whether the antibiotics directly

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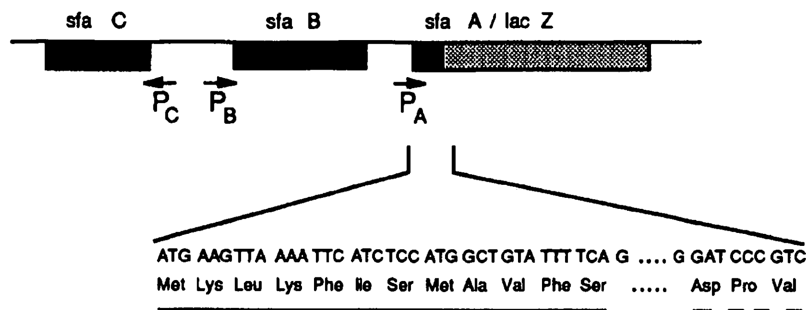

Fig. 1. Diagram of the proximal (5') part of the *sfa* determinant fused to the gene *lac*Z coding for β-galactosidase. The regulatory genes *sfa*C and *sfa*B and part of the structural gene *sfa*A are indicated by black boxes, *lac*Z part is indicated by a shadowed box. The promoter regions are marked by arrows. The DNA sequences and the deduced amino acid sequences of the 5' parts of *sfa*A are indicated by a solid line, *lac*Z sequences are marked by a dotted line. The junction site between *sfa*A and *lac*Z specific sequences is indicated.

repress or stimulate the transcription of the genes coding for bacterial products involved in pathogenicity factors (for review see [8]).

In general, pathogenicity factors like adhesins, toxins, capsules, iron uptake systems or IgA proteases enable bacteria to multiply in host organisms and to damage foreign tissues [9]. Pathogenic *E. coli* strains which are able to colonize the intestine (intestinal *E. coli*) or tissues outside the gut (extraintestinal *E. coli*) produce various pathogenicity factors [10,11]. Thus, *E. coli* strains responsible for extraintestinal infections like urinary tract infections (UTI), sepsis or new-born meningitis (NBM) [12] express hemolysins [13], capsule antigens [14], certain O antigens [11], iron uptake systems like aerobactin [15] and adhesins recognizing different eukaryotic receptor structures [12,16]. These pathogenicity factors contribute to the virulence of the strains [11,17].

One of these factors, the S fimbrial adhesin (Sfa) is produced by UTI and by NBM strains and is able to attach to sialic acid-containing receptor molecules [18–21]. We have cloned the genetic determinants coding for S fimbriae of NBM and UTI strains and have shown that nine genes including the gene *sfa*A responsible for the major structural protein are necessary for S fimbriae. The *sfa* determinant is transcribed by three different promoter regions ([22]; Fig. 1). Recently we have constructed a gene fusion between the gene *sfa*A of an *E. coli* wild-type pathogen and the gene *lac*Z coding for the enzyme β-galactosidase [23]. This wild-type fusion was used as a tool to determine the influence of environmental conditions on the expression of the *sfa* determinant.

In this study we have used the wild type gene fusion to measure the influence of low levels of a total of 28 antibiotics to gene expression of the *sfa* determinant. To our knowledge it is shown for the first time that various antimicrobial agents strongly influence gene expression of a virulence-associated gene cluster in a wild-type pathogen.

Materials and methods

Bacterial strains. The uropathogenic *E. coli* strain 536 WT (O6:K15:H31) exhibits various virulence factors including two hemolysins, S fimbriae and P-related fimbriae and is able to produce β-galactosidase (LacZ) as described in detail previously [24,25]. Strain 536-9B4/12 is an isogenic strain of 536 WT which carries a Tn5 insertion in the *lac*Z gene, leading to a non-functional original *lac* determinant. Furthermore, strain 536-9B4/12 carries a gene fusion between the *sfa* gene cluster coding for the S fimbrial adhesin and a second *lac*Z indicator gene which was introduced into strain 536 [23]. As shown by DNA sequencing, the *lac*Z gene is fused directly to base pair 37 of the 5' end of gene *sfa*A responsible for the structural subunit protein of the Sfa complex (see Fig. 1). The expression of *lac*Z which is under the control of the *sfa* wild-type promoters is equivalent to the expression of S fimbriae of strain 536.

Media, chemicals. Bacteria were grown in LB (Luria Bertani) broth or on LB agar plates with suitable amounts of antibiotics as described elsewhere

[8]. For detection of β-galactosidase production of the control strain, 536 WT plates with 0.05 mM isopropylthiogalactoside (IPTG) were used [26].

Antibiotics. The antibiotics used are listed in Table 1.

MIC determinations. The MIC determination was performed by microtiter plate assay DIN 58940, and the results are given in Table 1.

β-Galactosidase test. The *E. coli* strains were grown in LB broth overnight at 37°C to stationary phase. One ml of the culture was centrifuged and the bacteria were washed in 1 ml of 0.9% NaCl, and the suspension was finally adjusted to an OD_{600} of 0.6. Then the suspension was diluted 1:100 and 100 μl were plated on LB agar plates, containing the respective antibiotic concentrations. After growth overnight at 37°C, the bacteria were harvested in 0.9% NaCl and the suspension was adjusted to an OD_{600} of 1.0. 100 μl of this suspension was used for the β-galactosidase assay, that was performed according to Miller [26].

Statistics. Mean values ± standard deviation (s.d.) were calculated following the publication of Cavalli-Sforza [27].

Results

Antibacterial activities of antibiotics to strain 536 WT and 536-9B4/12. The uropathogenic strain 536 WT and its derivative 536-9B4/12 exhibited a chromosomally encoded resistance to streptomycin (*rps*L, see ref. [25]). As a consequence of the construction of the *sfa*A-*lac*Z wild-type gene fusion we introduced parts of the Tn*5* transposon which conferred resistance to kanamycin (*kan*) and an ampicillin resistance gene (*bla*) into the genome of strain 536-9B4/12 [23]. In addition, we determined the minimal inhibitory concentration (MIC) of another 28 antibiotics to strains 536 WT and 536-9B4/12. As shown in Table 1, the strains exhibited similar MIC values. They were highly resistant to 7 antimicrobial agents and were susceptible especially to the action of the monobactam antibiotic aztreonam, to cephalosporins and to quinolones (Table 1).

TABLE 1

Antibacterial activities of the antibiotics used

No.	Antibiotic (source)	MIC[1] (μg/ml) for strains	
		536 WT	536-9B4/12
1.	Imipenem (MSD, München, Germany)	0.25	0.25
2.	Ceftriaxone (Hoffmann-LaRoche, Basel, Switzerland)	0.063	0.063
3.	Cefotaxime (Hoechst, Frankfurt a.M., Germany)	0.063	0.063
4.	Cefodizime (Hoechst, Frankfurt a.M., Germany)	0.25	0.5
5.	Aztreonam (SQIBB, München, Germany)	0.063	0.125
6.	Cefalothin (Lilly, Bad Hamburg, Germany)	2	4
7.	Gentamicin (Serva, Heidelberg, Germany)	8	8
8.	Chloramphenicol (Serva, Heidelberg, Germany)	8	4
9.	Tetracycline (Serva, Heidelberg, Germany)	1	0.5
10.	Amphotericin B (SQIBB, München, Germany)	> 64	> 64
11.	Lincomycin (Sigma, Deisenhofen, Germany)	> 64	> 64
12.	Clindamycin (Upjohn, Heppenheim, Germany)	> 64	> 64
13.	Polymyxin B (Sigma, Deisenhofen, Germany)	1.25	1.25
14.	Fosfomycin (Boehringer, Mannheim, Germany)	4	4
15.	Sulfamethoxazole (Hoffmann-LaRoche, Basel, Switzerland)	> 64	64
16.	Trimethoprim (Sigma, Deisenhofen, Germany)	32	16
17.	Coumermycin (Hoffmann-LaRoche, Basel, Switzerland)	8	8
18.	Novobiocin (Sigma, Deisenhofen, Germany)	> 64	> 64
19.	Nalidixic acid (Serva, Heidelberg, Germany)	2	4
20.	Ciprofloxacin (Bayer, Leverkusen, Germany)	0.016	0.016
21.	Ofloxacin (Hoechst, Frankfurt a.M., Germany)	0.125	0.125
22.	Norfloxacin (Merck, Darmstadt, Germany)	0.125	0.125
23.	Rifampicin (Serva, Heidelberg, Germany)	4	2
24.	Nifurtimox (Bayer, Leverkusen, Germany)	> 64	> 64
25.	Niridazol (Ciba-Geigy, Basel, Switzerland)	16	16
26.	Nitrofurazone (Röhm-Pharma, Darmstadt, Germany)	16	16
27.	Tinidazole (Pfizer, Karlsruhe, Germany)	> 64	> 64
28.	Ornidazole (Hoffmann-LaRoche, Basel, Switzerland)	> 64	> 64

[1]MIC, minimal inhibitory concentration.

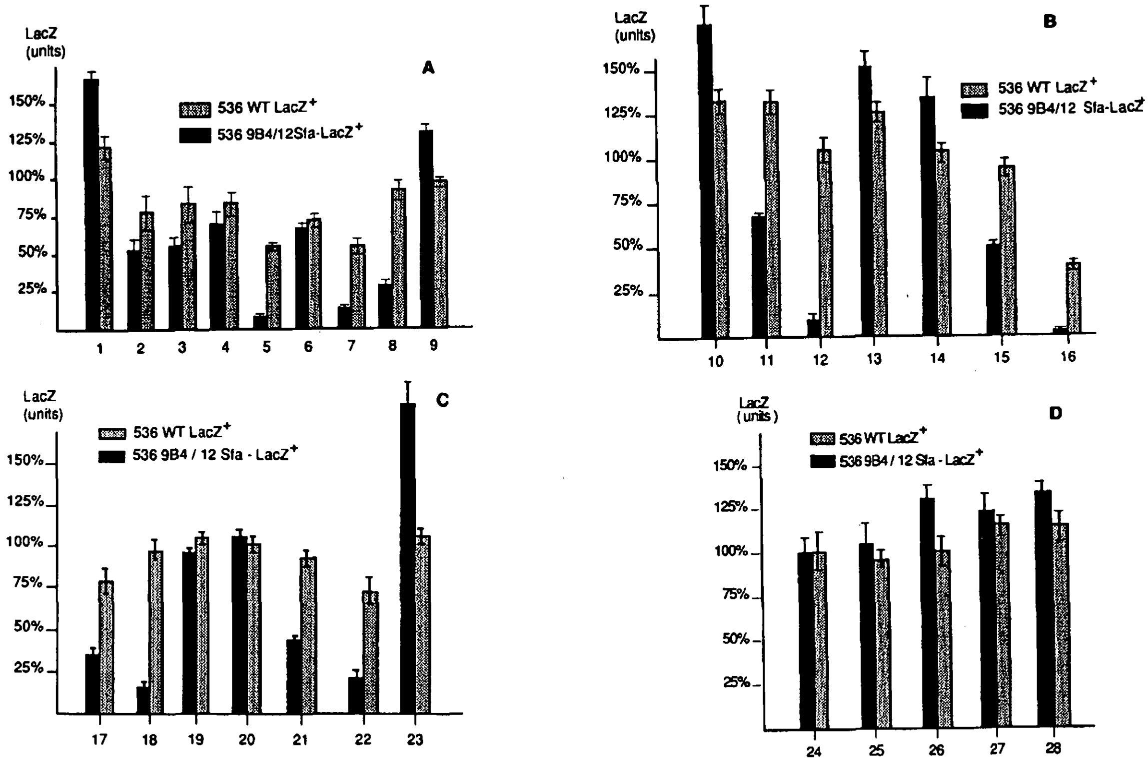

Fig. 2 A–D. Influence of 1/4 sub-MIC of antibiotics on β-galactosidase (LacZ) production of the wild-type strain 536 WT and the fusion strain 536-9B4/12 grown on solid medium. LacZ production by 536 WT was induced by addition of 0.05 mM IPTG to the growth medium. LacZ units are given as % values of the LacZ production after growing strains on plates without antibiotics which was set as 100%. The numbers indicate the antibiotics listed in Table 1.

Influence of sub-MIC on the expression of S fimbriae. In order to determine an influence of subinhibitory concentration (sub-MIC) on the expression of the *sfa* determinant, *E. coli* strains were grown on plates containing different amounts of antimicrobial agents and the LacZ values were measured. We used $^1/_4$ of the MIC values in the case of antibiotics which were active against the *E. coli* wild-type strains and 64 µg/ml in case of substances which did not act against the isolates. After cultivation of strains on plates without any antibiotics, strain 536 WT produced about 5000 LacZ units following induction of the *lac* operon with IPTG and strain 536-9B4/12 exhibited nearly 350 LacZ units (see ref. [23] and also Fig. 3). These values were set as 100% in Fig. 2. As shown in Fig. 2A, the monobactam aztreonam (7% LacZ units), the aminoglycoside gentamicin (12% LacZ units) and chloramphenicol (25% LacZ units) negatively influenced the expression of the *sfa* operon in strain 536-9B4/12. The expression of the *lac* operon in the control strain 536 WT was only weakly (aztreonam, gentamicin) or not influenced by the presence of the antibiotics. In contrast imipenem slightly stimulated *sfa* expression.

As shown in Fig. 2B, 64 µg/ml clindamycin (8% LacZ units) and in particular 4 µg/ml trimethoprim (2% LacZ units) had very strong negative effects on the expression of the *sfa* gene cluster. Other substances (e.g. amphotericin B) had weak or moderate positive effects on *sfa* expression. The quinolone derivatives indicated in Fig. 2C did not significantly influence the production of S fimbriae. Rifampicin had a moderate stimulating effect (170% LacZ units). It is additionally shown in Fig. 2D that nitro compounds did not have any effects on the expression of the *sfa* locus under aerobic growth conditions.

Influence of different trimethoprim and clindamycin

concentrations on sfa *expression.* As shown in Fig. 2, subinhibitory concentrations of both trimethoprim and clindamycin had suppressive effects on the expression of the *sfa* determinant. In order to determine the influence of the concentrations of the antimicrobial agents on *sfa* gene expression, the plates with different amounts of antibiotics were used for cultivation of strains 536 WT and 536- 9B4/12. As shown in Fig. 3A, 0.1 μg/ml trimethoprim reduces *sfa* expression from 320 LacZ units to 1–5 LacZ units. 0.025 μg/ml ($^1/_{500}$ of the MIC) still had an influence on *sfa* expression (120 units vs. 350 units without antibiotics). 10 μg/ml clindamycin had a weak effect on *sfa* expression but 30 μg/ml reduced the LacZ values from 350 to 50 units. A concentration of 40 μg/ml clindamycin reduced the expression of *sfa* to 10 LacZ units. The antibiotics had no (clindamycin) or only weak (trimethoprim) effects on *lac*Z expression of the control strain 536 WT.

Discussion

Subinhibitory concentrations of antimicrobial agents, i.e. concentrations below the minimal inhibitory concentration (MIC), have numerous effects on the bacterial cell. Such effects, unrelated to inhibition of growth, may include alterations of the virulence of strains (for review see [8,28]). These virulence alterations may result from secondary side effects, e.g. alteration of the cell morphology, decrease in the number of ribosomes [8,29], or from changes of the quantitative composition of the LPS, outer membrane proteins or crosslinking between OM and peptidoglycan [30–32]. On the other hand sub-MIC of antibiotics could also directly influence the production of pathogenicity factors as shown for the K1 antigen [3], hemagglutination [33] and P fimbriae of pathogenic *E. coli* [34], the M protein of streptococci [35], protein A of staphylococci [28] and various toxins of Gram-positive and Gram-negative bacteria (see ref. [8,36]). However, it is not known whether low doses of antibiotics directly suppress or stimulate the expression of the gene clusters encoding pathogenicity factors.

In the present study we used an *E. coli* wild-type gene fusion between the *sfa* determinant coding for S fimbriae and the indicator gene *lac*Z [23], by which we could show that sub-MIC of antibiotics like trimethoprim or clindamycin have a direct effect on the expression of the *sfa* gene cluster. Compared to other test systems, the strain used here has the advantage that, rather than the complex property 'adherence of bacteria' to eukaryotic cells including erythrocytes [5,33], simply the expression of one single adhesin gene cluster is evaluated.

In contrast to recombinant *E. coli* K-12 laboratory strains with cloned virulence determinants, our construct avoids undesirable multi-copy effects which could counteract regulatory events, since the *sfa*A-*lac*Z gene fusion is present in one copy on the chromosome. Additionally, wild-type strains differ from K-12 laboratory isolates in their genome structure as pathogenic strains carry large DNA regions (pathogenicity DNA islands) harboring additional pathogenicity determinants which may influence the expression of virulence-associated genes via *trans* regulatory pathways [16,17]. Furthermore, the wild-type pathogens differ from laboratory strains in several aspects including the composition of the O antigen and the presence of capsules [14,23]. Such constitutents of the cell surface highly influence the uptake of antimicrobial agents and the susceptibility of the bacterial cell and may therefore interfere with the effects of sub-MIC of antibiotics [8].

As indicated in Figs. 2 and 3, the antibiotics aztreonam, gentamicin, clindamycin and trimethoprim have the most pronounced effects on *sfa* gene expression. In all cases the *sfa* determinant is repressed (*sfa* promoter activity of 12% to 2%) following cultivation of strain 536-9B4/12 in the presence of these antibiotics. For trimethoprim it is shown that sub-MIC of $^1/_{128}$ (0.125 μg/ml) drastically affects *sfa* gene expression, lower doses of antibiotics lead to a less pronounced influence. In contrast to trimethoprim, which kills strain 536-9B4/12, the wild-type fusion strain is resistant to the action of clindamycin (see Table 1). The *sfa* determinant also is strongly repressed after growing the strain on medium containing 40 μg/ml clindamycin and *sfa* expression is still slightly affected by 10 μg/ml of this antibiotic.

It is difficult to speculate on the molecular mechanisms of the influence of the antibiotics on *sfa* gene expression, as the antimicrobial agents which repress *sfa* transcription show different modes of action. Trimethoprim acts as tetrahydrofolate-reduc-

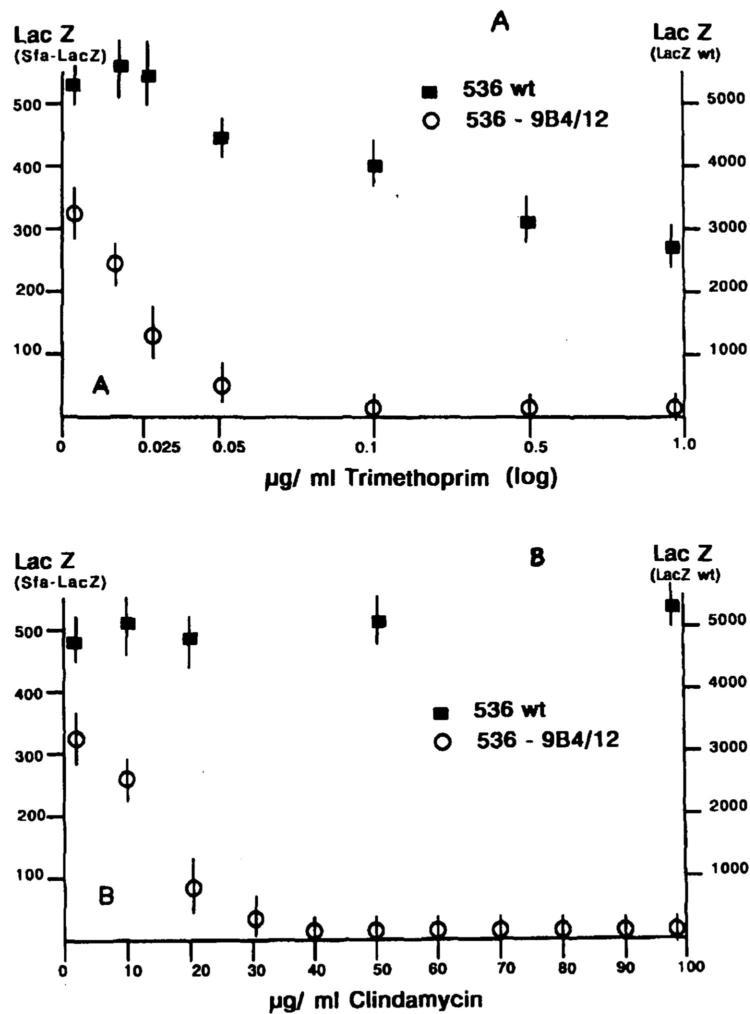

Fig. 3. Influence of different concentrations of trimethoprim (3A) and clindamycin (3B) on β-galactosidase (LacZ) production given as LacZ units, by the wild-type strain 536 WT and the fusion strain 536-9B4/12 grown on solid medium. LacZ production by 536 WT was induced by addition of 0.05 mM IPTG.

tase inhibitor, aztreonam represents a monobactam, which interferes with the murein synthesis, gentamicin is a aminoglycoside which acts like clindamycin on prokaryotic ribosomes. It is interesting to note that antibiotics which act on the DNA topoisomerase, like novobiocin, ciprofloxacin or ofloxacin (see Fig. 2C), and also the nitro compounds (Fig. 2D) had no observable effects on *sfa* expression.

It remains also be elucidated whether the antibiotics directly interfere with the promoter regions of the *sfa* determinant or whether they interact with the trans-regulator proteins SfaC and SfaB, which are necessary for the expression of S fimbriae [22]. Global transregulators like the osmo-regulator OsmZ also influence expression of virulence-associated genes including the *sfa* gene cluster [37], so that it cannot be excluded that antimicrobial agents interfere with these regulator proteins.

Studies on the influence of sub-MICs on the virulence of pathogenic strains share the problem that the clinical significance of the data is difficult to evaluate. In the case of urinary tract infections, however, trimethoprim in combination with sulfonamides is an antimicrobial agent commonly used in chemo-

therapy [38]. The finding that low doses of commonly used antibiotics influence the expression of major pathogenicity factors of pathogenic bacteria is of importance for the therapeutic setting.

Acknowledgements

The authors wish to thank E. Straube (Jena) and P. Koller (Frankfurt) for helpful discussions and U. Wallner (Würzburg) and M. Schmittroth (Würzburg) for excellent technical assistance. L.R. Phillips (Würzburg) is gratefully acknowledged for critical reading of the manuscript. The work was supported by a DFG grant (Ha 1434/1-7) and by the Fonds der Chemischen Industrie.

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