Group A streptococcal RofA-type global regulators exhibit a strain-specific genomic presence and regulation pattern

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RofA-like protein (RALP) type regulators have been shown to exist in different forms in group A streptococci (GAS) and to regulate the expression of important bacterial adhesins. This study shows that the vast majority of strains from different GAS M serotypes carried a *rofA* virulence regulator gene in their genome and that this gene could be detected in combination with other RALP genes and RALP-dependent adhesin genes in a strain-specific manner. The gene encoding the *Nra* regulator was predominantly found in opacity factor (OF)-negative serotypes. When analysing a *rofA* mutant in a serotype M2 strain, the strain specificity was also found in the positive and negative regulatory functions of RALP genes as well as in the type and number of virulence genes and functions controlled by the RALP genes. Of 17 virulence-associated genes tested, only one, the putative streptolysin S gene, was observed to be derepressed in RALP mutants of three different GAS serotype strains. This strain-specific variability of RALP regulon sizes is associated with different patterns of host cell attachment and internalization. In addition, RofA2 was shown to control expression of the ribosomal protein gene *rpsL*. As a consequence, it was demonstrated for the first time in streptococci that aminoglycoside resistance mediated by *rpsL* expression is apparently controlled by a virulence gene regulator.

Keywords: *Streptococcus pyogenes*, response regulator, virulence gene regulation, adherence and internalization, bacteria–host cell interaction

INTRODUCTION

Group A streptococci (GAS; *Streptococcus pyogenes*) cause a large variety of diseases in their human host, including suppurative infections of the pharynx and skin, and severe invasive manifestations. The organism can also exist in an asymptomatic carrier state. The ability to enter the host and colonize several different anatomical sites can be explained by co-ordinated and differentiated regulation of virulence factors in response to the local environment.

The recently completed genome sequence of serotype M1 revealed the presence of 13 potential two-component

signal transduction systems that may contribute to this differential regulation. Only six of these putative regulators can be assigned to specific functions based on sequence homology (Ferretti *et al.*, 2001). Of these, only the two sensor kinase/response regulator pairs CsrR/S (Levin & Wessels, 1998; Bernish & van de Rijn, 1999; Heath *et al.*, 1999; Federle *et al.*, 1999) and FasBCAX (Kreikemeyer *et al.*, 2001) have been studied in any detail. Besides the obvious sensor–regulator pairs, GAS encode a notably high number of global regulators without known cognate sensors.

The RopB protein is required for transcription of the gene encoding cysteine protease SpeB (Lyon *et al.*, 1998) and several other genes expressed primarily during stationary phase of growth (Chaussee *et al.*, 2001). Mga is a global positive transcriptional regulator that controls genes encoding the M- and M-related proteins, the fibronectin-binding serum opacity factor (OF), the

Abbreviations: GAS, group A streptococci; MSCRAMM, microbial surface components recognizing adhesive matrix molecules; OF, opacity factor; RALP, RofA-like protein.

cysteine protease SpeB, the C5a peptidase, the complement inhibiting factor Sic and the collagen-like proteins SclA and SclB (Perez-Casal *et al.*, 1991; Chen *et al.*, 1993; Podbielski *et al.*, 1995; McLandsborough & Cleary, 1995; Kreikemeyer *et al.*, 1999; Lukomski *et al.*, 2000a,b; Rasmussen *et al.*, 2000; Lukomski *et al.*, 2001; Rasmussen & Björck, 2001). The Mga regulator exists in two versions with about 70% sequence homology. The Mga type is associated with specific serotypes.

The RofA-like protein (RALP) regulator family comprises four different versions with a mean of 52% deduced amino acid sequence similarity and 29% identity (Granok *et al.*, 2000). Unlike the *mga* gene, several RALP regulators can be encoded by a single GAS strain (Podbielski *et al.*, 1999; Granok *et al.*, 2000; Ferretti *et al.*, 2001). However, the two best studied RALP-type regulators, RofA in a serotype M6 strain and Nra in a serotype M49 strain, occurred alone. RofA was first described as a positive transcriptional regulator of the gene encoding the fibronectin-binding protein (*prtF*) in GAS serotype M6 strains (Fogg *et al.*, 1994; Fogg & Caparon, 1997). RofA is autoregulated and responds to increased oxygen levels (VanHeyningen *et al.*, 1993). Since PrtF/SfbI is a major adhesin of GAS and also promotes internalization of the bacteria by epithelial cells (Hanski & Caparon, 1992; Talay *et al.*, 1991, 1992, 1994; Molinari *et al.*, 1997; Molinari & Chhatwal, 1999), RofA function is also important for bacteria–host cell interactions (Beckert *et al.*, 2001). As well as its positive regulatory effects, RofA was recently shown to suppress transcription of *speA*, *sagA* and *mga* (Beckert *et al.*, 2001). Therefore, RofA could be the repressor of *mga*, whose existence was recently suggested by McIver *et al.* (1999). Two RofA-binding boxes have been experimentally identified in the *rofA*–*prtF* intergenic region. Putative RofA-binding boxes were identified upstream of at least one other gene in the serotype M1 genome sequence (Granok *et al.*, 2000).

In contrast to RofA, the Nra pattern was shown to be that of a negative regulator in GAS. The *nra* gene is located adjacent to a collagen-binding adhesin (*cpa*) gene in serotype M49 strains (Podbielski *et al.*, 1999). The Nra regulon includes a gene encoding a second fibronectin-binding protein, PrtF2 (Jaffe *et al.*, 1996). Investigation of a small number of GAS serotypes indicated several combinations of *nra/rofA*, *prtF/prtF2* and *cpa* genes (Podbielski *et al.*, 1999). The type of genes regulated by RALP family members suggested an involvement in GAS–eukaryotic cell interactions exceeding the known functions of protein F. In fact, Nra regulation contributes to a balanced expression of virulence factors and avoidance of host cell damage, thereby promoting GAS persistence in eukaryotic cells (Molinari *et al.*, 2001).

It is possible that RALP family members could have more specific regulatory functions in particular serotypes than other GAS regulators. First, either one, two or several RALP genes can be found in combinations

with various MSCRAMM (microbial surface components recognizing adhesive matrix molecules; Patti *et al.*, 1994) genes like *prtF*, *prtF2* and *cpa*. Second, also the number and type of genes contained in a RALP regulon appear to be serotype-dependent.

To elucidate the presence of RALP-encoding genes and MSCRAMM-encoding genes in diverse GAS serotypes, 62 isolates were tested in the present study for the presence of *rofA*, *nra*, *prtF*, *prtF2* and *cpa* genes. Subsequently, a serotype M2 strain was chosen for functional analysis of its *rofA* gene because of the clinical importance of M2 strains e. g. in Germany (Brandt *et al.*, 2001) and because of the unusual structure of the core RALP regulon. To establish a nomenclature for *rof* and *nra* genes with respect to different M serotypes, the *rofA*-like gene in M2 was designated as *rofA2* from here on and *rofA*-like genes from other serotypes were designated accordingly. With three different serotypes now examined for the genes and functions under control of a single RALP-encoding gene, the differences between a common and the serotype-specific pattern of RALP function have begun to emerge.

METHODS

Bacterial strains and culture conditions. GAS strains were grown in THY at 37 °C in a 5% CO₂ atmosphere. The different M serotype GAS strains used in this study have been described by Podbielski *et al.* (1991, 1993) and Kaufhold *et al.* (1992). Production of serum OF was tested as described by Johnson & Kaplan (1988). All mutant strains were maintained on medium supplemented with 60 mg spectinomycin l⁻¹. The RofA6 and Nra49 mutant strains have been described elsewhere (Beckert *et al.*, 2001; Molinari *et al.*, 2001). The EmmLuc recombinant strain was constructed in this study according to methods published by Podbielski *et al.* (1999).

Nucleic acid techniques. Construction of the pSF152-*rofA6* plasmid used for insertional inactivation of the *rofA2* allele was described by Beckert *et al.* (2001). The Qiagen Mini Prep kit was used to prepare plasmid DNA from *Escherichia coli*. Preparation of competent *S. pyogenes* cells and transformation of GAS M2 followed the protocol of Caparon & Scott (1991). The presence of single plasmids in the M2 chromosome and the correct location of the inserted plasmid was confirmed by Southern blot techniques, PCR and Northern blot hybridizations. Chromosomal DNA from GAS was prepared according to Martin *et al.* (1990).

Genomic DNA of 62 serotype strains was digested with *EcoRI* or *HindIII* and, after agarose gel electrophoresis, blotted onto Pall Biodyne B membrane. For hybridization Dig-dUTP labelled PCR products were generated with primers described by Natanson *et al.* (1995), Jaffe *et al.* (1996) and Podbielski *et al.* (1999). In addition, the same primers were used for direct PCR assays using genomic DNA as template to amplify *rofA*, *nra*, *prtF*, *prtF2* and *cpa* genes. This analysis only included well characterized genes and not other so far uncharacterized RALP members identified by Granok *et al.* (2000).

Amplification of DNA by PCR was performed according to standard protocols (Ausubel *et al.*, 1990). For the generation of DNA probes for DNA–DNA and DNA–RNA hybrid-

ization, 0.5 µl DIG-dUTP was added to the reaction mixture. Agarose gel electrophoresis, DNA transfer to membranes, Southern blots and detection of hybridizing bands was carried out as described by Podbielski *et al.* (1995). The RNA preparation followed the protocol of Shaw & Clewell (1985). Denaturing agarose gel electrophoresis and Northern Blot hybridization with Dig-dUTP-labelled probes were as previously described (Podbielski *et al.*, 1995). DIG-dUTP-labelled gene probes of virulence factor and housekeeping genes for DNA-RNA hybridizations were generated by PCR, using various primers that were designed on the basis of published sequences.

Inverse PCR and sequencing. M2 chromosomal DNA (5 µg) was digested with *Hind*III. Digested DNA was purified according to standard methods (Ausubel *et al.*, 1990) and was used undiluted, and at dilutions of 1:10 and 1:100 for ligation. Ligation mixtures (10 µl total volume) were incubated overnight at 16 °C. Five microlitres of each ligation mixture were used as template for subsequent PCR amplification using primer RofAInvFor (5'-AGAACTTATCTTTACCAACTCTA-3') and RofAInvRev (5'-TTCGATTGATGATTCC-AAG-3'). The Expand High Fidelity system (Roche) was used and PCR conditions were as follows: 35 cycles each consisting of 1 min denaturation at 94 °C, 1 min annealing at 50 °C and 3 min 30 s extension at 72 °C. A 1.5 kb DNA band was amplified from all ligation mixtures. Sequencing of the purified regular and inverse PCR products was performed by MWG Biotech by utilizing the PCR primers as sequencing primers. Homology searches were performed with the global alignment algorithm described by Person *et al.* (1997).

Functional assays. Determination of haemolytic activity contained in supernatants of wild-type and RofA2 mutant bacteria and measurement of binding capacity towards extracellular matrix and plasma proteins was performed as described previously (Kreikemeyer *et al.*, 2001). Culture and maintenance of the human laryngeal epithelial cell line HEP-2 (ATCC CCL23,) and adherence and invasion assays using wild-type and mutant bacteria were essentially as described by Molinari *et al.* (2001). For all functional assays described above three individual tests were performed on three different days. Data shown are the means of such assays with standard deviations ($\pm$ SD) indicated. Determination of MICs employed the E test according to the manufacturer's instructions (AB Biodisk).

Statistical analyses. The Mann-Whitney U test was used to determine if the mean values of the datasets from the functional assays are significantly different. The significance level (α) at which the test was performed is indicated in the main text of the appropriate section.

RESULTS

Presence of RALP and MSCRAMM genes in 61 GAS serotypes

To examine the occurrence of different RALP- and MSCRAMM-encoding genes in a collection of diverse GAS serotypes we applied PCR and hybridization techniques to chromosomal DNA isolated from 62 strains (representing 61 different M serotypes). All MSCRAMM-encoding genes known to be members of RALP regulons were included (*prtF*, *prtF2* and *cpa*). However, for RALP-encoding genes the study focussed only on the functionally characterized members (*rofA6* and *nra49*) and excluded the uncharacterized *ralP3*

and *ralP5* genes (Granok *et al.*, 2000). The data are summarized in Table 1.

Fifty-two (83 %) of the strains tested harboured a *rofA* gene, whereas only 17 (27 %) carried the *nra* gene. Of these 17 strains, seven (41 %) contained both *rofA* and *nra* genes. Only three OF-positive strains carried a copy of *nra*. In contrast, fourteen OF-negative strains were *nra*-positive, suggesting *nra* to be more common among OF-negative strains. The *prtF* gene was present in 70 % of the strains tested. A comparable frequency as well as the association of *prtF* with OF-positive serotypes was also reported by Natanson *et al.* (1995) and Goodfellow *et al.* (2000). A total of 53 % of the strains carried a *prtF2* gene and 32 % harboured a *cpa* gene. The majority of strains (52 out of 62) carried one or two MSCRAMM-encoding genes. No MSCRAMM gene was detected in serotype M2, M3, M52 and M57 strains.

All possible combinations of RALP- and MSCRAMM-encoding genes were found. Additionally, no association was detected between RALP/MSCRAMM patterns and specific diseases commonly associated with particular GAS serotypes.

Construction of a *rofA* mutant in the GAS serotype M2

Based on the epidemiological importance of this serotype (Brandt *et al.*, 2001), the presence of only a *rofA* gene, and the unusual RALP- and MSCRAMM-encoding gene pattern, an M2 serotype strain was chosen for further characterization to investigate the strain specificity of RALP-mediated regulation. Using regular and inverse PCR strategies we were able to amplify the *rofA2* gene and to determine its sequence. The sequence showed a 98.3 % identity with the *rofA6* gene sequence (accession number U01312; Fogg *et al.*, 1994). The corresponding *nra49* gene sequence (accession number U49397; Podbielski *et al.*, 1999) exhibited only 62.6 % sequence homology. The *rofA2* gene was inactivated by insertion mutagenesis (Beckert *et al.*, 2001) and the mutation was confirmed by PCR and Southern hybridization (data not shown). No differences in the growth rate and final optical cell density between wild-type and mutant was detected (data not shown).

Characterization of extracellular matrix- and plasma-protein binding activity of the M2 wild-type and RofA mutant

One of the main features of RALP family members is their involvement in transcriptional regulation of fibronectin binding (M6 and M49) and collagen-binding adhesins (M49). Expression of these MSCRAMMs directly influences important biological functions, such as bacteria-host cell interactions.

Therefore, we investigated the extracellular and plasma protein-binding capacity of the wild-type M2 strain and the RofA2 mutant at the early exponential, late exponential and stationary phases of growth (Table 2). Only low levels of fibrinogen binding were observed for

Table 1. Distribution of RALP- (*rofA* and *nra*) and MSCRAMM- (*prtF*, *prtF2* and *cpa*) encoding genes among 62 strains of 61 different M serotypes

–, Absence; +, presence of the gene, as judged by PCR and Southern hybridization experiments. For details of the assay refer to Methods. OF column: +, positive; –, negative. Numbers in bold depict total number of strains positive for the tested gene, and in parenthesis the percentage of the total strains tested is given.

M serotype	OF	<i>rofA</i>	<i>nra</i>	<i>prtF</i>	<i>prtF2</i>	<i>cpa</i>
1	–	+	–	–	–	+
2	+	+	–	–	–	–
3	–	–	+	–	–	–
4	+	+	+	+	+	–
5	–	–	+	–	+	–
6	–	+	–	+	–	–
6	–	+	–	+	–	–
8	+	+	–	–	–	+
9	+	+	–	+	+	+
11	+	+	–	+	+	+
12	–	+	–	+	+	–
13	+	+	–	+	+	–
14	–	+	–	+	+	+
15	+	+	–	+	–	–
17	–	+	–	+	–	–
18	–	–	+	–	+	–
19	–	+	–	+	–	–
22	+	+	–	+	+	+
23	–	+	–	+	–	–
24	–	+	–	+	–	–
25	+	+	–	+	–	+
26	–	+	–	+	–	–
27	–	+	–	+	–	–
28	+	+	–	+	+	–
29	–	+	–	+	–	+
30	–	+	–	+	–	+
31	–	+	–	+	–	–
32	–	+	+	+	+	+
33	–	–	+	–	+	–
34	–	–	+	–	+	–
36	–	+	+	–	+	+
37	–	+	–	+	–	–
38	–	+	–	+	–	–
39	–	–	+	+	+	–
40	+	+	–	+	–	–
41	–	+	–	+	–	+
42	–	–	+	–	+	+
43	–	–	+	–	+	+
44	+	+	–	+	+	–
46	–	+	–	+	–	–
47	–	+	–	+	+	+
48	+	+	–	+	–	–
49	+	–	+	–	+	+
50	–	+	–	+	+	–
51	–	+	–	+	+	–
52	–	+	+	–	–	–
53	–	+	+	–	+	–
54	–	+	–	+	–	–
55	–	+	–	+	+	–

Table 1 (cont.)

M serotype	OF	<i>rofA</i>	<i>nra</i>	<i>prtF</i>	<i>prtF2</i>	<i>cpa</i>
56	—	+	+	—	+	+
57	—	+	+	—	—	—
58	+	+	—	+	—	—
59	+	—	+	+	+	—
60	+	+	—	+	+	—
61	+	+	—	+	+	—
62	+	+	—	+	+	—
65	—	+	—	+	—	—
66	+	+	—	+	+	—
67	—	+	—	—	+	+
69	—	+	—	—	+	+
71	—	+	—	—	+	+
None-typeable (64/14)	—	+	—	+	—	—
Total: 62	+ 20 — 42	52 (83 %)	17 (27 %)	43 (70 %)	33 (53 %)	20 (32 %)

Table 2. Extracellular and plasma protein binding by a serotype M2 GAS strain and its isogenic RofA2 mutant

The assay was performed as described by Kreikemeyer *et al.* (1995). Results from three independent assays are shown. The Mann–Whitney U test was used to calculate statistical significance. The percentage binding was calculated as bound c.p.m. $\times$ 100/added c.p.m.

Protein and strain type		Percentage binding*		
		3 h	8 h	Overnight
Fibronectin	Wild-type	22.3 $\pm$ 1.2	29.1 $\pm$ 2.1	19.1 $\pm$ 0.9
	Mutant	23.3 $\pm$ 0.4	14.1 $\pm$ 0.8	14.9 $\pm$ 0.6
Fibrinogen	Wild-type	9.5 $\pm$ 0.9	9.5 $\pm$ 0.9	7.3 $\pm$ 0.4
	Mutant	11.3 $\pm$ 0.1	7.5 $\pm$ 0.4	6.5 $\pm$ 0.3
Collagen I	Wild-type	32.1 $\pm$ 3.4	42.8 $\pm$ 0.6	41.1 $\pm$ 0.2
	Mutant	41 $\pm$ 0.6	38.8 $\pm$ 1.3	39.9 $\pm$ 1.8

* Mean $\pm$ SD.

the wild-type and mutant at any phase of growth. In contrast, high level binding of collagen type I was detected. The *rofA2* mutation increased collagen binding by 10 % in early exponential phase. However, the difference was not statistically significant (α , 5 %) and the binding activity of both the wild-type and mutant were the same during the later stages of growth. Binding of fibronectin was not significantly different in the wild-type and mutant bacteria from early exponential phase, but the RofA2 mutant showed a significant (α , 5 %) 50 % decrease in fibronectin binding during late exponential phase. In cells harvested from late stationary phase cultures a significant difference in binding capacity was still detectable. These results suggested that RofA in M2 acted as a positive regulator of fibronectin-binding activity in agreement with earlier observations with the RofA6 mutant (Fogg *et al.*, 1994; Beckert *et al.*, 2001).

The PCR and Southern blotting analyses described in Table 1 indicated that the well characterized *prtF* and *prtF2* genes encoding fibronectin-binding proteins were absent from strain M2. Therefore we performed PCR analyses and Southern hybridization experiments using primers and probes directed against *fbp54* and *sof/sfbII*. These two MSCRAMM-encoding genes have been shown to encode proteins capable of fibronectin binding (Rakonjac *et al.*, 1995; Kreikemeyer *et al.*, 1995, 1999; Courtney *et al.*, 1994, 1999; Katerov *et al.*, 2000), and might therefore be responsible for the fibronectin-binding activity observed in this serotype. With both experimental approaches the presence of *fbp54* and *sof/sfbII* genes was verified (data not shown). It has never been investigated if either of these genes is controlled by a RALP-type regulator. Strains lacking *PrtF* and *PrtF2* have been described (Natanson *et al.*,

Table 3. Summary of transcriptional analysis of genes in defined RofA/Nra mutants

The data are accumulated from published results (Fogg *et al.*, 1994; Podbielski *et al.*, 1999; Beckert *et al.*, 2001; Molinari *et al.*, 2001) and this study. 0, transcription not affected; –, decreased transcription; +, increased transcription; NT, not tested; NP, not present in serotype.

RALP mutant	Housekeeping genes		Surface expressed virulence factors							Secreted virulence factors				Regulatory genes					
	<i>recA</i>	<i>rpsL</i>	<i>cpa</i>	<i>fbp54</i>	<i>emm</i>	<i>prtF2</i>	<i>prtF</i>	<i>sof</i>	<i>hasB</i>	<i>speA</i>	<i>speB</i>	<i>sagA</i>	<i>slo</i>	<i>ska</i>	<i>fasX</i>	<i>mga</i>	<i>nra</i>	<i>csr</i>	<i>rofA</i>
Nra M49	NT	NT	+	+	0	+	NP	+	0	+	+	+	0	0	NT	+	+	NT	NP
RofA M6	NT	NT	NP	NT	+	NP	–	NP	NT	+	0	+	0	NT	NT	+	NP	NT	–
RofA M2	0	–	NP	0	–	NP	NP	0	–	0	0	+	0	+	0	0	NP	0	–

Fig. 1. Northern blot transcriptional analysis of the serotype M2 GAS strain (wild-type; WT) and the isogenic RofA2 mutant strain (mutant). For preparation, quantification, electrophoresis, hybridization and detection of RNA refer to Kreikemeyer *et al.* (2001). DNA probes used are indicated above each part. Results shown are representative of those from at least three independent RNA isolations. The amounts (µg) of RNA loaded in each lane are indicated above each lane. Molecular masses (kb) of the RNA bands are indicated on the left hand side of each panel.

1995), but were not studied with respect to RALP regulation.

These results raised the question about the genomic environment of the *rofA* gene in the M2 serotype, since in other serotypes either *prtF* or *cpa* have been found

adjacent to the *rofA* gene. To test this, the region between the 5' end of the *lepA* gene (putative signal peptidase I; Podbielski *et al.*, 1999) and the 5' end of the *rofA2* gene was amplified as a fragment of 600 bp suggesting the absence of *prtF* or *cpa* genes. A similar experiment using a *lepA* and *nra* specific primer pair on

Table 4. Antibiotic resistance measured by the E test (AB Biodisk)

The RofA2 mutant and EmmLuc strains were constructed in this study; all other strains are described by Podbielski *et al.* (1999), Beckert *et al.* (2001) and Molinari *et al.* (2001). The data shown are representative of three independent assays. NT, Not tested. Values represent mg l⁻¹

GAS strain	Gentamicin	Streptomycin
M2 WT	16	32
RofA2 mutant	96	64
M6 WT	32	NT
RofA6 mutant	16	NT
M49 WT	12	NT
Nra49 mutant	12	NT
EmmLuc	12	NT

M49 DNA generated a 3.3 kb fragment, consistent with the presence of *cpa* in this serotype (data not shown).

Effects of *rofA* inactivation on expression of selected genes and biological functions

The RALP family members in serotype M6 and M49 control a variety of virulence genes at the transcriptional level (Podbielski *et al.*, 1999; Beckert *et al.*, 2001) (see also Table 3). Therefore, our next objective was to determine how the *rofA* mutation in serotype M2 would influence gene expression (Fig. 1, Table 3). No truncated *rofA2* message was detected in the mutant strain, indicating an at least 8-fold lower transcription in the mutant (Fig. 1a). This suggested a positive auto-regulatory effect of RofA2, a fact that was also noted in the RofA6 mutant (Beckert *et al.*, 2001). The genes encoding M protein (*emm*; Fig. 1b), capsule synthesis enzymes (*hasB*; Fig. 1c), and ribosomal small subunit protein S12 (*rpsL*; Fig. 1h) were also found to be under positive control of RofA in serotype M2. RofA6 and RofA2 both acted as negative regulators of *mga* transcription (Beckert *et al.*, 2001; Fig. 1j), but unlike in M6, *emm* transcription in M2 appears not to be directly coupled to transcription of *mga*, since contrasting effects on *emm* were observed in the two serotypes.

In GAS, *rpsL* has been used as a constitutively expressed control gene (Federle *et al.*, 1999). Mutations in *rpsL* caused resistance to, and in some cases dependence on, streptomycin (Toivonen *et al.*, 1999; Björkman *et al.*, 1999). Thus, we investigated how downregulation of *rpsL* affected aminoglycoside resistance in the wild-type and mutant strain. Table 4 shows that downregulation of *rpsL* caused increased MIC values for gentamicin and streptomycin in the RofA2 mutant. This effect can be explained by the decreasing number of target sites for aminoglycosides in the RofA2 mutant and is independent of the spectinomycin resistance determinant used for generating the mutant, since the MICs for gentamicin in the unrelated spectinomycin-resistant mutants were unaltered (Table 4).

The genes encoding the two secreted virulence factors, streptokinase (*ska*) and streptolysin S (*pel/sagA/sls*), were under negative transcriptional control of RofA in strain M2 (Fig. 1f, g). Enhanced expression of the gene encoding streptolysin S was verified in a functional assay. The supernatant of the RofA2 mutant contained a significantly (α , 5%) higher haemolytic activity ($148 \pm 6.8\%$) compared to the M2 wild-type bacteria ($100 \pm 4.5\%$), with the haemolytic activity contained in supernatants from wild-type arbitrarily set to 100%. Transcription of *speA* in strain M2 was not affected by the *rofA2* mutation (Fig. 1i), in contrast to serotypes M6 and M49, where RofA6 and Nra49, respectively, caused *speA* to be transcribed at higher levels, suggesting negative control. Judged from their unchanged expression levels, *recA*, *fasX* (Fig. 1d, e), *csrR/S* and the gene encoding SpeB (data not shown) are not part of the *rofA2* regulon. Of note, the fibronectin-binding proteins encoded by *fbp54* and *sof2* did not appear to be regulated by RofA2 (Fig. 1k, l), suggesting an unidentified fibronectin-binding mechanism that is RALP-dependent. A comparison of the housekeeping and virulence gene transcription levels in RofA or Nra mutants from M2, M6 and M49 is summarized in Table 3.

Influence of the *rofA* mutation on eukaryotic cell adherence and internalization

Adherence to and internalization into cultured epithelial cells *in vitro* is a well established feature of *S. pyogenes* (Molinari & Chhatwal, 1999). The processes are mediated by a fibronectin bridge which links bacterial cells to integrins on the epithelial cell surface and induces macro-pinocytosis (Molinari *et al.*, 1997; Okada *et al.*, 1998; Ozeri *et al.*, 1998). Focal complex-like structures and recruitment of kinases from at least two signalling pathways facilitate the invasion process (Ozeri *et al.*, 2001). Results presented in Table 2 suggest that RofA2 is a positive regulator of fibronectin binding. If this is true, the RofA2 mutant would be less adhesive and invasive compared to the wild-type bacteria. Therefore, we investigated the influence of the RofA2 regulatory effects on cell adherence and internalization by performing antibiotic protection assays as described previously (Molinari *et al.*, 2001). Unexpectedly, the RofA2 mutant was significantly (α , 1%) more adherent to the cultured HEp-2 cells than the wild-type strain ($187 \pm 55\%$ for the mutant; wild-type adherence was arbitrarily set to 100%). In contrast, the internalization rate of the mutant into HEp-2 cells compared to the wild-type was not significantly changed ($1.41 \pm 0.84\%$ for the mutant; $0.9 \pm 0.5\%$ for the wild-type).

DISCUSSION

The study described in this paper had two major objectives. First, we intended to examine the occurrence of different RALP- and MSCRAMM-encoding genes in a diverse collection of different GAS serotypes to determine the most frequent patterns of gene com-

binations. Second, we intended to find common patterns of regulation by RALP-encoding genes in order to predict the association of patterns of RALP/MSCRAMM genes and biological properties of specific GAS serotype strains.

The present investigation revealed that the most frequent representative of RALP genes is *rofA*, which was present in 83 % of the GAS strains tested. An *nra*-like gene was found in 27 % of the strains. In two-thirds of these strains *nra* and *rofA* occurred together. Each of the two RALP-encoding genes, either alone or in combination with other RALP-encoding genes, occurred in combination with one, two or even three RALP-dependent MSCRAMM-encoding genes. Based on the analysis of 61 different GAS serotypes there is no obvious pattern of RALP- and RALP-dependent MSCRAMM-encoding genes. This observation also applies to any potential link between gene patterns and the association of GAS serotypes with non-suppurative diseases like acute rheumatic fever or acute glomerulonephritis. Based upon the detection of anti-PrtF antibodies in sera from GAS-infected patients, Goodfellow *et al.* (2000) were also unable to demonstrate any association between antibody levels and the type of infection. We detected the *prtF* gene in 70 % of the isolates tested. This number is in agreement with earlier reports by Kreikemeyer *et al.* (1995), Natanson *et al.* (1995) and Goodfellow *et al.* (2000), who reported 83 %, 66 % and 64 % of isolates, respectively, to be *prtF* positive. In accordance with Goodfellow *et al.* (2000) we demonstrated a positive association between the occurrence of a *prtF* gene and the expression of serum OF. The only OF-positive strains without a detectable *prtF* gene were the serotype M2, M8 and M49 strains, of which the more thoroughly characterized M2 strain was the only one without any detectable MSCRAMM-encoding gene. Taken together, the presence or absence of specific RALP- or MSCRAMM-encoding genes seems to have little influence on the capacity of a GAS strain to cause purulent or non-suppurative diseases. The high number of detectable combinations among the different types of genes in the various serotypes indicates an ongoing and efficient lateral transfer of these genes.

To approach the second major objective of this study, an analysis of the *rofA* function in the serotype M2 strain using insertion mutagenesis appeared to be most promising. Downstream effects potentially caused by the insertion appeared very unlikely due to the monocistronic transcription mode of the *rofA2* allele and presence of transcription terminators on the inserted vector. However, collateral damage caused by the *rofA2* mutation could not be completely excluded, since complementation experiments were not attempted in this study. *In trans* complementation of response regulator mutants often leads to potentially ambiguous results due to gene dosage effects. In fact, functional analysis of a *nra49* mutant (Molinari *et al.*, 2001) that was complemented *in trans* did not result in restoration of the wild-type phenotype but in different gene expression patterns (B. Kreikemeyer & A. Podbielski,

unpublished results). Genetic systems for *in cis* chromosomal reconstitution are currently not available for GAS.

So far, the presence of a RALP-dependent MSCRAMM gene adjacent to the cognate RALP-encoding gene was regarded as a key feature of the genomic environment of this regulator family. Using different experimental approaches, we showed that the *prtF* and *cpa* genes did not lie upstream of the *rofA* locus in the M2 serotype strain. Thus, such a strain was best suited when investigating the minimum congruency between RALP functions in a variety of GAS strains. Regardless of the absent *prtF* or *cpa* gene, the M2 wild-type strain exhibited strong collagen type I- and fibronectin-binding, indicating the presence of other surface factors with matrix-protein-binding capacity in the M2 strain. This also demonstrated a functional redundancy among MSCRAMMs. Judged from the increased binding profile of the RofA2 mutant, these factors were also under control of RofA. The deduced simultaneous positive and negative effects on the expression of various genes resembles the *rofA6* but not the *nra49* regulatory profile.

The MSCRAMMs responsible for the observed binding profile remain obscure. The M2 strain was shown to possess a *sof* and a *fbp54* gene, both of which have been associated with fibronectin binding (Rakonjac *et al.*, 1995; Kreikemeyer *et al.*, 1995, 1999; Courtney *et al.*, 1994, 1999; Katerov *et al.*, 2000). However, transcription of both genes was unaltered in the RofA2 mutant. So far Cpa is the only collagen-binding MSCRAMM in GAS that has been characterized at the molecular level. Since M2 lacks Cpa it is likely that the M2 strain studied here harbours a novel collagen-binding mechanism.

Fibronectin binding due to protein F has been unequivocally associated with host cell attachment and internalization. Similarly, derepression or repression of fibronectin binding by protein F in Nra49 or RofA6 mutants led to increased or decreased cell attachment and internalization, respectively. However, the RofA2 mutant behaved unexpectedly. Despite its decreased fibronectin-binding activity it showed increased cell attachment and unchanged internalization. GAS host cell attachment and internalization independent of the *prtF* genes has been demonstrated before (Jaffe *et al.*, 1996; Molinari *et al.*, 2000, 2001). The present study indicates that such *prtF*-independent mechanisms do not rely on a structural coupling of both processes, as it was demonstrated for the protein F function (Talay *et al.*, 2001). The factors responsible for the increased affinity of the RofA2 mutant for HEp-2 cells remain uncharacterized. However, the decreased expression of genes involved in capsule synthesis in the mutant could contribute to the observed effects. Inactivation of the *has* operon in the M2 strain increased its adherence to keratinocytes by a factor of two (Darmstadt *et al.*, 2000).

In summary, in three different GAS serotype strains examined for their RALP regulation pattern, differences were found in the expression of all but one virulence gene. Only *sagA*, putatively encoding streptolysin S, was

consistently derepressed in the RofA2, RofA6 and Nra49 mutants. Thus, not only the distribution of RALP genes and RALP-dependent MSCRAMM genes, but also the expression patterns of many virulence genes contained in RALP regulons exhibit a strain-specific variability. This variability clearly exceeds that of the regulons of the global Mga and CsrR regulators.

A potential association of this variability with the pathogenic potential of a given GAS serotype cannot be excluded in spite of the inconclusive results of the present investigation. However, a definitive answer necessitates the collection and molecular characterization of a very large number of GAS strains together with the information about their clinical background.

The analysis of *rofA2*-dependent gene expression resulted in an interesting side aspect. So far, *rpsL*-like ribosomal protein genes were assumed to be constitutively expressed. Now we could demonstrate that in GAS, *rpsL* transcription is under control of RofA2. Concerning the mutant's resistance to aminoglycosides, the decreased RpsL expression had the same effect as mutations in *rpsL* (Brakier-Gingras & Phoenix, 1984) potentially by decreasing the number of targets for the antibiotics. However, changes in surface characteristics leading to decreased uptake of antibiotics may also account for the increased MIC.

This is the first demonstration of altered antibiotic resistance levels due to the activity of a virulence regulator in streptococci.

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