

Quorum-sensing regulation of a capsular polysaccharide receptor for the *Rhodobacter capsulatus* gene transfer agent (RcGTA)

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Summary

The gene transfer agent produced by *Rhodobacter capsulatus* (RcGTA) resembles a small tailed bacteriophage that packages almost random genomic DNA segments that may be transferred to other *R. capsulatus* cells. Gene transfer agents are produced by a number of prokaryotes; however, no receptors have been identified. We investigated the RcGTA recipient capability of wild-type *R. capsulatus* cells at different culture growth phases, and found that the frequency of RcGTA-dependent acquisition of an allele increases as cultures enter the stationary phase. We also found that RcGTA adsorption to cells follows a similar trend. RcGTA recipient capability and adsorption were found to be dependent on the GtaR/I quorum-sensing (QS) system. Production of an extracellular polysaccharide was found to be regulated by GtaR/I QS, as was production of the cell capsule. A number of QS-regulated putative polysaccharide biosynthesis genes were identified, and mutagenesis of two of these genes, *rcc01081* and *rcc01932*, yielded strains that lack a capsule. Furthermore, these mutants were impaired in RcGTA recipient capability and adsorption, as was a non-encapsulated wild-type isolate of *R. capsulatus*. Overall, our results indicate that capsular polysaccharide is a receptor for the gene transfer agent of *R. capsulatus*, RcGTA.

Introduction

The term gene transfer agent (GTA) is used for genetic exchange elements that resemble bacteriophages, but unlike bacteriophages, exclusively package almost random fragments of the host cell genome into a transducing particle (Humphrey *et al.*, 1997; Eiserling *et al.*, 1999; Hynes *et al.*, 2012; Lang *et al.*, 2012). The size of DNA in the particles is smaller than the DNA encoding the GTA structural proteins, thus a single particle is unable to transfer the genes required for its own synthesis to a new host (Lang *et al.*, 2012). This is in contrast to generalized transducing bacteriophage, where the DNA molecule packaged is equivalent in size to the phage genome, and the frequency of packaging of host cell DNA is very low

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Supporting information

Additional supporting information may be found in the online version of this article.

(Ikeda and Tomizawa, 1965). Numerous phylogenetically distinct bacteria produce functional GTA particles, including the α -proteobacterium *Desulfovibrio desulfuricans* (the GTA Dd1) (Rapp and Wall, 1987), the spirochaete *Brachyspira hydroseriae* (VSH-1) (Matson *et al.*, 2005), the archaeon *Methanococcus voltae* (VTA) (Eiserling *et al.*, 1999), and the α -proteobacterium *Rhodobacter capsulatus* (RcGTA) (Marrs, 1974; Lang *et al.*, 2012).

A model system for studying GTAs is *R. capsulatus* RcGTA. This GTA may have evolved from a bacteriophage soon after the α -proteobacteria branched from the other proteobacteria, because RcGTA gene homologues appear to have been inherited vertically as part of the cell genome in a wide variety of species (Lang and Beatty, 2007). The RcGTA resembles a small tailed bacteriophage that packages ~ 4.3 kb of donor cell genomic DNA (Yen *et al.*, 1979) that can be transduced in to an *R. capsulatus* recipient cell and integrated into genome, most likely through homologous recombination (Genthner and Wall, 1984). The RcGTA structural genes are encoded by a ~ 15 kb gene cluster, ruling out the possibility of self-propagation by a single RcGTA particle (Lang and Beatty, 2000). In addition to the RcGTA main cluster, genes required for regulation, release and infectivity have been found in distant genome locations (Chen *et al.*, 2009; Lang *et al.*, 2012).

Production and release of RcGTA is dependent on a number of factors, including growth phase (Florizone, 2006), phosphate concentration (Westbye *et al.*, manuscript in preparation), and a putative phosphorelay system involving homologues of the *Caulobacter crescentus* cell cycle regulatory proteins CtrA, ChpT and CckA (Lang and Beatty, 2000; Mercer *et al.*, 2010). An additional regulatory system depends on the GtaR and GtaI quorum-sensing (QS) proteins (Schaefer *et al.*, 2002; Leung *et al.*, 2012). GtaR and GtaI are homologous to LuxR- and LuxI-like proteins (Lazdunski *et al.*, 2004) respectively, where LuxI-like proteins synthesize *N*-acyl-homoserine lactone (acyl-HSL) signalling molecules, and LuxR-like proteins function as cognate receptors that bind these signals and regulate transcription. LuxI/R-type QS systems regulate a variety of typically group-oriented bacterial behaviours, such as the pathogenicity of bacteria infecting plants (Piper *et al.*, 1993) or humans (Winson *et al.*, 1995; Williams *et al.*, 2000; Bottomley *et al.*, 2007), stem-nodule symbiosis in plants (Daniels *et al.*, 2002; Tun-Garrido *et al.*, 2003), horizontal gene transfer (Piper *et al.*, 1993; Leung *et al.*, 2012), extracellular polysaccharide (EPS) production and biofilm formation (Davies *et al.*, 1998; Gonzalez and Keshavan, 2006; Nadell *et al.*, 2008). Depending on the system, transcription of target genes may be induced or repressed when a threshold concentration of acyl-HSL is reached (Lazdunski *et al.*, 2004). The GtaI protein synthesizes two long-chain acyl-HSLs, C14 and C16-acyl-HSL, either of which causes the induction of RcGTA production (Schaefer *et al.*, 2002), and the production of RcGTA is stimulated by several acyl-HSLs that are not produced by *R. capsulatus* itself (Leung *et al.*, 2012). The GtaR protein functions as a negative regulator of the *gtaRI* operon in the absence of acyl-HSLs synthesized by GtaI, and GtaR modulates the production of RcGTA by indirectly repressing RcGTA transcription in the absence of acyl-HSL (Leung, 2010; Leung *et al.*, 2012).

Although the structure, regulation and genetics of RcGTA have been studied for decades, the identity of the bacterial receptor used for RcGTA attachment has been a mystery. Many surface-exposed structures of Gram-negative bacteria, including lipopolysaccharide (LPS),

outer membrane proteins, and EPS such as a cell capsule function as phage receptors (Choy *et al.*, 1975; Rakhuba *et al.*, 2010). For example, it was reported that a phage-resistant *R. capsulatus* strain lacked a capsule, indicating a role of the bacterial capsule in the adsorption of that phage (Flammann and Weckesser, 1984).

In this study, we investigated factors affecting the ability of *R. capsulatus* to act as a recipient for RcGTA-mediated gene transduction. Through a variety of analyses, we found that capsule synthesis in *R. capsulatus* is quorum-sensing regulated, and that the capsule polysaccharide functions in RcGTA adsorption during gene transduction, and so is the first example of a GTA receptor.

Results

RcGTA recipient capability and attachment of RcGTA to *R. capsulatus* cells are growth phase-dependent

We investigated the effect of growth phase on RcGTA recipient capability of *R. capsulatus* cells by quantifying the number of cells that received and expressed a rifampicin-resistance marker from a stock solution of RcGTA of known titre, using cells harvested at different time points during culture growth (Fig. 1A). The recipient capability of *R. capsulatus* cells at early growth phases was much less than those at high cell densities, showing a positive correlation between the optical density (OD) of cultures at time of harvest and recipient capability (Fig. 1B). The recipient capability does not address whether cells in early growth phases lack an RcGTA receptor molecule, the required recombination machinery, or other possible factors required for RcGTA-mediated gene transduction. We investigated the possibility of a receptor deficiency by employing an RcGTA adsorption assay with the same culture samples used as RcGTA recipients. The ability of RcGTA to adsorb to cells was measured by quantifying the transduction efficiency of cell-free (non-adsorbed) RcGTA after incubation with cells. It was found that the ability of cells to adsorb RcGTA increased in tandem with the culture density of the culture at time of harvest, indicating that cells increased their ability to adsorb RcGTA particles in later growth phases (Fig. 1C).

These two types of experiment show that the ability of cells to bind RcGTA particles and acquire a new allele is culture growth phase-dependent, and maximal in the stationary phase. By analogy with the attachment of bacteriophage to cell surface-exposed receptors, we hypothesized that a receptor required for RcGTA binding is produced, or becomes available for attachment, as cultures increase in cell concentration.

Effects of mutations in *gtaR* and *gtaI* on RcGTA recipient capability and attachment to cells

RcGTA production is regulated by the *gtaR* and *gtaI* QS genes (Schaefer *et al.*, 2002; Leung *et al.*, 2012). The correlations of the increases in recipient capability and RcGTA cell-adsorption to culture density indicated a possible link to the GtaR/I QS system. To evaluate this possible link, we determined the recipient capability of stationary phase WT, *gtaI*, *gtaR* and *gtaRI* cells, as well as the *gtaI* mutant supplemented with C16-acyl-HSL. It was found that the *gtaI* mutant yielded on average 7% as many gene recipients as the WT strain, and recipient capability was restored to 109% of the WT level by the addition of

exogenous C16-acyl-HSL (Fig. 2A). Additionally, we observed that the *gtaR* and *gtaRI* mutants yielded average recipient frequencies of 103% and 98% of WT respectively (Fig. 2A).

To determine whether the decrease in the *gtaI* mutant recipient capability was a result of decreased binding of RcGTA to cells, we performed an adsorption assay on all the cultures evaluated in the recipient capability experiments described above. The *gtaI* mutant was greatly impaired in the adsorption of RcGTA particles, and adsorption was restored to WT levels by the addition of C16-acyl-HSL (Fig. 2B). Furthermore, WT amounts of RcGTA adsorption were observed for the *gtaR* and *gtaRI* mutants, with the amounts of cell-free RcGTA inversely proportional to the recipient capability of these mutants (Fig. 2B). In Western blots probed for the RcGTA capsid protein remaining in the filtrate of the adsorption assay, the amounts of capsid paralleled the residual RcGTA activity values, confirming that the amount of RcGTA had decreased (Fig. 2C). These results indicate that GtaR/I QS regulates the expression of genes encoding a cell surface factor required for RcGTA adsorption to cells.

Analysis of EPS production in *gtaR* and *gtaI* mutant strains

Rhodobacter capsulatus—WT strain B10 cells form a loose cell pellet when centrifuged and this pellet is easily dissociated with a single inversion of the tube (Fig. 3A). The *gtaI* mutant, under the same conditions, produces a tight pellet that requires considerable mixing force to re-suspend. The *gtaR* mutant has a loose pellet phenotype, as does the *gtaRI* double mutant (Fig. 3A). These results indicate that one or more genes required for the loose pellet phenotype is negatively regulated by the GtaR protein, because disruption of the *gtaR* gene does not change this phenotype, whereas mutation of the *gtaI* gene suppresses the effects of the *gtaI* mutation.

The loose cell pellet persisted after several washes in 50 mM NaCl, indicating that the looseness of the cell pellet is a property due to a substance that is tightly associated with cells (data not shown). To evaluate the causative agent of the loose cell pellet phenotype, WT cells were treated with a selection of enzymes or EDTA. We found that treatment with EDTA, which is known to disrupt the outer membrane of Gram-negative bacteria (Hancock, 1984), or proteinase K, resulted in a tight cell pellet whereas other chemicals and enzymes tested did not have a similar effect (Fig. S1). These results indicate that the loose pellet phenotype is due to a cellular structure attached to the outer membrane of *R. capsulatus* B10 cells, and it may involve a protein component. In other bacteria, similar cell pellet phenotypes are caused by EPS such as capsular polysaccharide (Ionescu and Belkin, 2009). Because *R. capsulatus* B10 has a capsule (Weaver *et al.*, 1975), we hypothesized that the loose pellet was caused by the production of an EPS that is part of the capsule.

We roughly quantified the total amount of EPS and LPS in EDTA extracts of cells using a phenol-sulfuric acid method and found that the levels of cell-associated EPS/LPS correlate with the loose pellet phenotype (Fig. 3B). The *gtaI* strain produced much less EPS/LPS than WT cells, and this production was restored by addition of exogenous C16-acyl-HSL. Furthermore, both the *gtaR* and *gtaRI* mutants produced WT levels of EPS/LPS (Fig. 3B). These results indicate that the GtaR/GtaI QS system regulates EPS/LPS production, but

do not determine whether this EPS/LPS is the *R. capsulatus* capsule, or whether the loose cell pellet phenotype relates to the binding of RcGTA.

There was a consistently high background signal when quantifying surface polysaccharides with the phenol-sulfuric acid method. EDTA extracts contain many molecules associated with the outer membrane. Because the phenol-sulfuric acid assay measures total carbohydrate levels, the consistently high background measurements are likely a result of non-capsular polysaccharide (such as LPS) being present in extracts of strains lacking the capsule.

Microarray and bioinformatic identification of putative EPS biosynthesis genes

To identify genes potentially involved in the production of EPS/LPS, we utilized microarrays to evaluate the relative gene expression in the *gtaI* mutant versus WT cells [NCBI Gene Expression Omnibus (GEO) database Accession No. GSE41014]. A number of potential polysaccharide biosynthesis genes were downregulated in the *gtaI* mutant, between 2.1- and 3.1-fold in the stationary phase (Table 1). We selected a putative polysaccharide biosynthesis operon, containing open reading frames (ORFs) *rcc01081-rcc01086* as candidates for the production of an EPS. The *rcc01081-rcc01086* ORFs (Fig. 4) are annotated in the *R. capsulatus* strain SB1003 genome as two group 1 glycosyltransferases (GTase), an epimerase, two putative membrane proteins, and a group 1 GTase respectively (summarized in Table 2). To more accurately annotate these ORFs, we performed BLASTp searches and hydropathicity analyses (Whitfield, 2006; Moreno-Hagelsieb and Latimer, 2008). The top BLASTp hits to Rcc01081-Rcc01086 include components present in both LPS O-antigen biosynthesis systems and capsular polysaccharide biosynthesis operons (Cuthbertson *et al.*, 2009; Raetz and Whitfield, 2002; Whitfield, 2006). These include three GTases, an epimerase, and genes predicted to encode a polysaccharide flippase (*wzx*) and a polymerase (*wzy*) (Table 2). Hydropathicity analysis of the Rcc01081-Rcc01086 proteins lends support to these annotations, notably the Wzx (encoded by *rcc01084*) and Wzy (*rcc01085*) protein homologues. Although the amino acid sequence conservation of Wzx and Wzy proteins is often low, a hydropathy profile of multiple transmembrane segments is a strongly conserved characteristic (Whitfield, 2006). The genes we suggest to encode Wzx- and Wzy-like proteins have a hydropathy profile indicating eight to nine transmembrane segments (Table 2 and Fig. S2).

To verify that that these genes are QS-regulated, we measured the promoter activity of an *rcc01081::lacZ* in-frame fusion plasmid, which contains approximately 1.5 kb of sequences 5' of the *rcc01081* start codon. The WT, *gtaI*, *gtaR*, *gtaRI* strains containing this plasmid were grown phototrophically in RCV defined medium to the stationary phase, and the β -galactosidase specific activity measured. We found that *rcc01081::lacZ* expression in the *gtaI* strain was reduced to 25% of the WT activity, and that expression was restored to WT levels upon addition of C16-acyl-HSL to cultures (Fig. S3). Furthermore, both the *gtaR* and *gtaRI* strains yielded WT levels of *rcc01081::lacZ* expression (Fig. S3), verifying that *rcc01081* expression is GtaR/GtaI-regulated.

The foregoing analysis prompted us to search the *R. capsulatus* genome for homologues of the initiating GTases WbaP and WecA, as well as Wzb, Wzc and Wza homologues, which

function as machinery for polymer export to the cell surface (Whitfield, 2006; Cuthbertson *et al.*, 2009). A BLASTp search identified ORFs *rcc01932*, encoding a WecA homologue (34% amino acid identity), and *rcc01958-1960*, which encode homologues of: Wzc (Rcc01958); Wzb (Rcc01959); and Wza (Rcc01960); (38%, 41% and 33% amino acid identity respectively) (Table 2). The hydrophathy profile of Rcc01932 matched the profile expected for this protein (Fig. S2). The *rcc01932* gene (encoding the putative initiating GTase, WecA) is of particular interest because it is downregulated under the same conditions as the *rcc01081-1086* cluster, and could encode an enzyme catalysing a key step in polysaccharide chain biosynthesis. On the basis of these analyses, we hypothesized that the *rcc01081-rcc01086* and *rcc01932* genes are involved in capsule production in *R. capsulatus*, and that this capsular polysaccharide is important for RcGTA adsorption to cells.

Analysis of capsule production and mutagenesis of *rcc01081* and *rcc01932*

Capsule production in the *gtaI*, *gtaR*, *gtaRI* mutants, and *gtaI* supplemented with C16-acyl-HSL, was evaluated using a capsule stain (Anthony, 1931) and microscopic examination of cells. We found that WT *R. capsulatus* B10 cells have a clear halo surrounding the cells (Fig. 5C), indicative of the presence of a capsule. The *gtaI* mutant lacks this halo, indicating the lack of a capsule. Capsule production was restored in the *gtaI* mutant by addition of C16-acyl-HSL; furthermore, both the *gtaR* and *gtaRI* strains produced capsules similar to those of WT cells. These results indicate that the GtaR protein negatively regulates production of the capsule, analogous to the GtaR-regulation of RcGTA production (Leung *et al.*, 2012) and recipient capability.

To test the hypothesis that the ORFs *rcc01081-1086* are involved in capsular polysaccharide production, we generated a marker-less, in-frame knockout mutation of the first ORF in the cluster, *rcc01081*, which is annotated as a group 1 glycosyltransferase. A capsule stain of *1081* mutant cells showed that they do not produce an extracellular capsule and the cells are weakly stained with crystal violet/copper sulphate (Fig. 5C). The *1081* mutation was observed to cause gross cell defects: cultures form a large aggregate of cells when grown phototrophically in RCV medium, which associate as a floating mass when agitated (Fig. 5B). After centrifugation, the *1081* cells form a tight cell pellet similar to that of *gtaI* cells, and produce a low amount of EPS as measured in the phenol-sulfuric acid assay (Fig. 3A and B). Phase contrast microscopy revealed that *1081* cells are irregular in shape, filamentous, and appear as large aggregates, in contrast to the WT morphology (Fig. 5A). The *1081* mutant was complemented *in trans*, and the complemented strain, *1081*[p1081], was phenotypically similar to the WT in liquid culture growth, cell morphology, formation of a cell pellet, levels of EPS, and production of a capsule (Figs 3 and 5).

The microarray and promoter::*lacZ* analysis indicated that the *rcc01081-1086* gene cluster is downregulated in the *gtaI* mutant (Table 1 and Fig. S3). If the phenotype of the *1081* mutant strain is due to a block in a polysaccharide biosynthesis pathway that is downregulated in the *gtaI* strain, a *gtaI/1081* double mutant would be expected to lack the gross morphological defects of the *1081* mutant. We therefore generated a marker-less double mutant *gtaI/1081*. The cell morphology, growth, pellet formation, levels of EPS and capsule of the double mutant were similar to those of the *gtaI* mutant in liquid culture

(Figs 3 and 5). Furthermore, supplementation of *gtaI*/*1081* cultures with C16-acyl-HSL resulted in a phenotype that was similar to that of a *1081* single mutant: cells aggregated, yielded tight pellets, produced a low amount of EPS, and lacked a capsule (Figs 3 and 5).

To evaluate whether *rcc01932*, encoding the predicted WecA homologue, is involved in capsule production, we generated a marker-less, in-frame knockout of this gene. Capsule stains of *1932* mutant cells show that they lack a capsule (Fig. 5C). Additionally, the *1932* mutant produces less EPS than the WT strain as measured by the phenol-sulfuric acid method, and forms a tight pellet after centrifugation (Fig. 3). Unlike the *1081* mutant, however, *1932* cells are morphologically normal when viewed in a microscope (Fig. 5A). When grown phototrophically in RCV defined medium, *1932* cells clump to a lesser extent than *1081* mutant cells, and the *1932* cell mass disintegrates readily with agitation (Fig. 5B). The *1932* mutation was complemented *in trans* in an equivalent fashion to the *1081* mutation, and the complemented strain *1932*[p1932] was phenotypically similar to WT cells (Figs 3 and 5). To provide additional evidence that *rcc01932* and *rcc01081* function in the same capsule biosynthesis system, a double mutant of *rcc01081* and *rcc01932* was created. The *1932*/*1081* strain showed a similar phenotype to the *1932* single mutant (Figs 3 and 5), indicating that mutation of *rcc01932* suppresses the morphological defect of the *1081* mutant, and provides evidence that these two genes function in the same capsule biosynthesis system.

To confirm that the foregoing results were consistent with a capsule biosynthesis defect, we compared our mutant strains with *R. capsulatus* strain 37b4, a non-encapsulated WT isolate (Omar *et al.*, 1983). The 37b4 strain pelleted tightly, produced less EPS than WT B10 cells, and did not have a capsule halo (Figs 3 and 5). The properties of strain 37b4 were essentially identical to the *gtaI* strain phenotype, supporting the idea that the EPS described here is indeed a capsule polysaccharide.

Recipient capability of and RcGTA attachment to strains *1081*, *1932* and 37b4

We tested the RcGTA recipient capability and adsorption ability of the strains *1081*, *1081*[p1081], *gtaI*/*1081*, *gtaI*/*1081* supplemented with C16-acyl-HSL, *1932*, *1932*[p1932], *1932*/*1081*, and 37b4. It was found that the *1081*, *gtaI*/*1081*, *1932*, and *1932*/*1081* mutants were greatly impaired in recipient capability, yielding on average 10%, 6%, 4% and 1% respectively, as many transductants as the WT strain B10. This defect was somewhat restored in the *1081*[p1081] complemented strain, which yielded 58% of the WT level (Fig. 6A), and fully restored in the *1932*[p1932] strain, which yielded 136% of the WT level. Unlike the *gtaI* mutant, however, recipient capability was not restored to the *gtaI*/*1081* mutant by addition of C16-acyl-HSL (Fig. 6A), supporting the interpretation that the defect in recipient capability and attachment in the *gtaI* strain involves downregulation of the *rcc01081-1086* genes. Additionally, the considerable reduction in recipient capability of the *1932* strain indicates that this gene is also involved in the production of the capsule. This interpretation was confirmed by RcGTA adsorption assays which showed that the *1081* and *1932*, and *1932*/*1081* mutants were impaired in RcGTA attachment. As in the recipient capability experiments, RcGTA-adsorption was not restored in the *gtaI*/*1081* double mutant by the addition of C16-acyl-HSL (Fig. 6B

and C). To confirm the interpretation that the capsule is required for RcGTA attachment, we tested the 37b4 strain for recipient capability. Attempts at transduction into 37b4 were unsuccessful, and this strain was effectively unable to adsorb RcGTA (Fig. 6). These observations indicate that production of a capsule polysaccharide requires the *rcc01081-1086* and *rcc01932* genes, and that the capsule is needed for maximal adsorption of RcGTA – and hence, acquisition of alleles originating in another cell.

Inhibition of RcGTA adsorption by surface polysaccharide extracts

Although our results indicate that the *rcc01081* and *rcc01932* are involved in capsule polysaccharide biosynthesis, mutagenesis of such genes can also cause pleiotropic effects (as is seen in the *1081* mutant), and may interfere in other biosynthetic pathways such as LPS O-antigen biosynthesis. Because LPS is often used as a bacteriophage receptor, we investigated the possibility that the reduced RcGTA adsorption in our mutants was due to an altered LPS structure. We visualized the LPS of all our strains by silver staining (data not shown), and did not observe differences in the banding pattern in any of our B10-derived mutant strains, indicating that these strains produce a WT LPS. However, there was a difference in LPS banding of the 37b4 strain. As a further step, we evaluated whether surface extracts from the WT B10 encapsulated strain, but not from non-encapsulated mutants, inhibit RcGTA adsorption to cells. It was found that addition of exogenous WT B10 extracts, but not extracts from the *gtaI* or *1932* strains (both of which lack a capsule), significantly blocked RcGTA adsorption to cells (Fig. 7A and B), supporting the conclusion that capsular polysaccharide is involved in RcGTA adsorption.

Discussion

We report the discovery of a receptor involved in the adsorption of RcGTA to *R. capsulatus* cells. The receptor is an EPS that we ascribe to the capsule layer surrounding most strains of *R. capsulatus* (Weaver *et al.*, 1975). This finding has interesting implications in terms of horizontal gene transfer in laboratory and environmental contexts, as well as possibly in biotechnological applications by extending the host range of GTAs.

The initial observation of changes in RcGTA recipient capability and attachment of *R. capsulatus* B10 cells in different growth phases prompted us to evaluate whether the GtaR/I QS system is involved in the process. As hypothesized, we found that RcGTA recipient ability and adsorption are negatively regulated by the GtaR protein, as was shown for RcGTA production (Leung *et al.*, 2012). The regulation by the GtaR/I system of not only the production of RcGTA particles in donor cells, but also the display of an RcGTA receptor on the surface of recipient cells indicates a long and complex evolutionary history. We speculate that part of the natural selection for QS regulation of this RcGTA receptor stems from an advantage gained by restraining the energetically expensive production of the receptor polysaccharide unless potential RcGTA donors are present in the environment, as indicated by the presence of the acyl-HSL signal. Similarly, the ultimate cost associated with the production of RcGTA particles (cell death) would provide no benefit to kin unless there were nearby cells potentially capable of taking up RcGTA.

The *rcc01081-1086* gene cluster has similarities to both group 1 LPS O-antigen biosynthesis genes and group 1 or 4 capsule biosynthesis genes. When sequence analysis alone cannot reliably distinguish between O-antigen, capsular polysaccharide or other EPS biosynthesis genes, genomic location may provide some insight (Whitfield, 2006). LPS O-antigen biosynthesis clusters are typically located in close proximity to other biosynthesis genes, such as those that synthesize the core oligosaccharide (Raetz and Whitfield, 2002); however, no such genes were found in the proximity of *rcc01081-1086*. For group 4 capsules, the genes required to synthesize a polysaccharide repeat unit usually are separate from the other required genes. For example, in *Escherichia coli*, functions additional to the synthesis of a repeat unit, such as polysaccharide chain initiation and export, are carried out by functional homologues in other gene clusters, such as the '22 minute' locus (Whitfield, 2006). Our search for the required chain initiation and export machinery yielded clear homologues of such proteins encoded in the *R. capsulatus* genome, namely Rcc01932 (WecA; chain initiator), and Rcc01958-1960 (Wzc, Wzb and Wza; export). Furthermore, the pattern of regulation of these genes is similar to that of the *rcc01081-1086* genes (Table 1). Based on this information, and the fact that most *R. capsulatus* strains have a capsule, we hypothesized that the *rcc01081-1086* gene cluster is involved in capsule biosynthesis.

Initially, we were surprised by the phenotype of the *1081* strain, as it forms a massive aggregate when grown in RCV defined medium and large clumps of swollen, filamentous cells when viewed in the microscope. We also observed that the *rcc01081* mutant strain pellets well, and produces a level of cell-associated EPS similar to the *gtaI* and *37b4* strains, consistent with a defect in the same EPS biosynthetic pathway. We ascribe the aggregation of strain *1081* misshapen cells to the build-up of an intracellular polymer (Clarke *et al.*, 2004). It is possible that this polymer is assembled on an polyisoprenoid lipid undecaprenyl diphosphate (und-PP) carrier that is also used in peptidoglycan biosynthesis (Xayarath and Yother, 2007), and so this phenotype may be the result of a defective peptidoglycan layer due to the continual unproductive occupancy of an und-PP carrier. The weak staining of *1081* cells with crystal violet (Fig. 5C) is consistent with a defect in peptidoglycan production. Future experiments aimed at identifying the precise function of the Rcc01081 protein may include a comparative analysis of EPS from WT and *1081* strains, as was done by others to propose the sugar composition of the *R. capsulatus* capsule (Omar *et al.*, 1983).

Loss of function mutagenesis of *rcc01932* indicated that this gene is involved in the same pathway as the *rcc01081-1086* genes, because cells neither produce a capsule nor adsorb RCGTA effectively. Unlike the *1081* strain, however, *1932* mutant cells have a near-normal morphology (Fig. 5). Because the Rcc01932 protein is most likely involved in polysaccharide chain initiation, we predicted that mutation of the *rcc01932* gene would result in a strain lacking a particular EPS. Indeed, the phenotype of the *1932* mutant strain strengthens the suggestion of a group 4-like capsule biosynthesis system, because mutagenesis of a gene in a distant genomic location from the *rcc01081-1086* cluster generated a phenotype similar to that of the *1081* mutant. Additionally, the *1081/1932* double mutant has a phenotype similar to that of the *1932* single mutant, providing strong evidence that these two distantly linked genes function in the same capsule biosynthesis

system (i.e. mutagenesis of *rcc01932* suppresses the cell shape defect caused by the *rcc01081* mutation).

A previous analysis of the active RcGTA recipient population in a culture found that nearly all cells were active recipients (Solioz *et al.*, 1975). Our capsule stains indicated that all cells in a population produce a capsule under the conditions tested, and so our results are in good agreement that the entire population can act as RcGTA recipients, whereas RcGTA is produced by a sub-population of cells (Fogg *et al.*, 2012; Hynes *et al.*, 2012).

It is interesting that the genes *rcc01079* and *rcc01080*, adjacent to the *rcc01081-1086* cluster (Fig. 4), encode putative tail fibre proteins that co-purified with RcGTA particles in a proteomic study (Chen *et al.*, 2009). Other experiments indicated that *rcc01079* and *rcc01080* are required for maximal RcGTA-mediated transduction frequencies (Lang *et al.*, 2012). Thus it appears that RcGTA receptor and receptor-binding genes are adjacent on the chromosome, and separate from the structural gene cluster (*rcc01682-01698*).

Many bacteriophages that target capsular polysaccharide as a receptor, such as coliphage K5a, K1-5, and the Vi phages of *Salmonella enterica*, possess enzymes that degrade capsular polysaccharide upon binding (Gupta *et al.*, 1983; Scholl *et al.*, 2001; Pickard *et al.*, 2010). Such a mechanism allows the phage to traverse the capsule layer, which can span a relatively large distance (~ 0.5 µm). One mechanistic example is the Kf1A protein of coliphage K5a, which was shown to function as a polysaccharide lyase that degrades K5 capsular polysaccharide, and is a tail-spike protein (Clarke *et al.*, 2000). It is possible that RcGTA uses a similar mechanism to traverse the capsule layer and deliver DNA into cells. It was previously noted that RcGTA orf *g15* has weak similarity to a putative rhamnosyl transferase and that rhamnose is a component of the *R. capsulatus* capsule (Lang and Beatty, 2000).

In a previous study, it was reported that the capsular polysaccharide of *R. capsulatus* strain St Louis was initially obtained as part of an SDS-solution-insoluble complex. This complex was found to contain peptidoglycan, protein and capsular polysaccharide, and the capsular polysaccharide was liberated by addition of a protease (Bräutigam *et al.*, 1988). Furthermore, the same complex purified from the non-encapsulated strain 37b4 was found to lack the polysaccharide component attributed to the capsule, although the protein-peptidoglycan complex remained; the same complex purified from a phage-resistant strain of St Louis, RC1-, was also found to lack attached capsular polysaccharide (Bräutigam *et al.*, 1988). These findings are of note for two reasons. First, the fact that capsular polysaccharide attachment involved a protein component may explain our observation that proteinase K abolishes the loose pellet phenotype. Second, it is an interesting parallel that an *R. capsulatus* genuine phage also appears to utilize the capsule as a receptor in a fashion analogous to RcGTA.

The discovery of a receptor involved in RcGTA adsorption is an important step forward in terms of understanding how and when RcGTA-mediated genetic exchange functions. It may also provide insight into how *R. capsulatus* interacts with its own species and in mixed species communities. Now that a receptor involved in adsorption has been discovered, the groundwork for further characterization of exactly how the RcGTA attaches to and transfers

DNA into cells has been established, and may allow for future application in gene transduction between species.

In summary, we propose that the capsule polysaccharide of *R. capsulatus* is a receptor involved in RcGTA adsorption to cells, and that production of the capsule is dependent on culture growth phase and the GtaR/I QS system. We identified genes that are required for capsule production and demonstrated that their expression is dependent on QS. Our findings show that the regulation of production of an RcGTA receptor as well as the production of RcGTA itself are controlled by the same QS system. These results indicate that RcGTA, an RcGTA receptor, and QS genes have evolved in concert. We anticipate that these findings will lead to additional development of RcGTA as a tool and a better understanding of the function of RcGTA and other GTAs in natural settings.

Experimental procedures

Bacterial strains and growth of cultures

The *E. coli* strains DH5 α and S17-1 (Simon *et al.*, 1983) were used for cloning and conjugation of plasmids into *R. capsulatus* respectively. *E. coli* strains were grown at 37°C in Luria–Bertani (LB) medium (Sambrook *et al.*, 1989) supplemented with the appropriate antibiotics at the following concentrations ($\mu\text{g ml}^{-1}$): ampicillin, 150; gentamicin sulphate, 10; kanamycin sulphate, 50.

The *R. capsulatus* strains B10 (WT) (Marrs, 1974), *gtaI*, *gtaR*, *gtaRI*, *1081*, *1081*[p1081], *gtaI/ 1081*, *1932*, *1932*[p1932], *1932/ 1081* and 37b4 (WT) (Weckesser *et al.*, 1972) were grown at 30°C in defined RCV medium (Beatty and Gest, 1981) phototrophically for EPS measurements, culture images, capsule-staining, and β -galactosidase assays. For RcGTA adsorption assays, cells were grown aerobically in RCV medium with shaking (flask filled to 50% of nominal capacity) at 200 r.p.m. With the exception of the growth curve experiment, cells were harvested in the stationary phase for use in assays. When appropriate, media were supplemented with ($\mu\text{g ml}^{-1}$): gentamicin sulphate, 3; kanamycin sulphate, 10; rifampicin, 80, and tetracycline, 1.

Culture turbidity was used as a measure of the number of cells per ml, and monitored in a Klett-Summerson photometer (red filter #66); 100 Klett units represents approximately 4×10^8 *R. capsulatus* colony-forming units per ml (cfu ml^{-1}). For RcGTA recipient capability and adsorption assays, cell turbidity was measured at 660 nm in a spectrophotometer; an OD₆₆₀ of 1 = $\sim 4.5 \times 10^8$ cfu ml^{-1} .

Recombinant DNA techniques and plasmids

Standard methods of DNA purification, restriction enzyme digestion, and other modification techniques were used (Sambrook *et al.*, 1989). The plasmid pUC19 (Invitrogen) was used for subcloning, pIND4 (Ind *et al.*, 2009) as an expression vector for the *rcc01081* and *rcc01932* complementation experiments, and pXCA601 (Adams *et al.*, 1989) for the *rcc01081* promoter *lacZ* fusion experiments.

The plasmids used in this work are listed in Table S1. The *rcc01081::lacZ* promoter fusion plasmid containing ~ 1.5 kb of sequence 5' of the *rcc01081* start codon was used to evaluate transcription and translation of the *rcc01081* gene. The insert was made by PCR amplification using WT *R. capsulatus* genomic DNA as a template, and the primer set 1081_lacZ_for and 1081_lacZ_rev. The resultant amplicon was digested with PstI and BglII, and ligated into pXCA601. The ligation resulted in a translationally in-frame fusion between the *rcc01081* promoter sequences and the *lacZ* gene.

Creation of the suicide vector pZDJ

Generating silent knockouts in *R. capsulatus* using the suicide plasmid pZJD29a (J. Jiang and C.E. Bauer, unpublished) is relatively inefficient due to recombination of the *puc* promoter region into the *R. capsulatus* chromosome. Additionally, counter-selection efficiency on sucrose is low, as the *puc* promoter does not effectively induce the expression of the *sacB* gene under aerobic conditions. We thus created a new suicide vector, pZDJ, created by replacing the *puc* promoter with the *tetA* promoter, allowing for constitutive expression of the *sacB* gene, and to allow for use of this vector in other Gram-negative bacteria, such as *Rhodobacter sphaeroides*.

Briefly, pZJD29a was linearized with ApaI and inverse PCR amplified using the primer set sacB:accC1_for and sacB:accC1_rev. This reaction yielded an ApaLI to MfeI fragment, with the *R. capsulatus puc* promoter removed. The *tetA* promoter from the broad-host range vector pRK415 was amplified using the primers tetAp_for and tetAp_rev with modifications from (Larsen *et al.*, 2002), generating an MfeI to ApaLI fragment. These two PCR fragments were digested with ApaLI and MfeI, ligated together, and transformed into *E. coli* DH5 α λ pir, resulting in the 4.8 kb suicide vector pZDJ (Fig. S4).

Construction of chromosomal deletion mutant strains

All gene disruptions made in this study were markerless in-frame deletions of the majority of the gene. All mutant strains were constructed from the *R. capsulatus* B10 strain. The *gtaI*, *gtaR*, and *gtaRI* strains were constructed as described previously (Leung *et al.*, 2012). The *1081* mutant was constructed by amplifying ~ 700 bp flanking regions of the *rcc01081* gene using the primer pairs 1081_up_for and 1081_up_rev, and 1081_down_for and 1081_down_rev. The PCR products were inserted into the vector pUC19 as a Sall-HindIII fragment (upstream) and a HindIII-SacI fragment (downstream). The resultant vectors were then digested with the appropriate enzymes, ligated together to generate a Sall-SacI fragment, and inserted into the suicide plasmid pZDJ. This vector was conjugated into *R. capsulatus* B10 and allowed to recombine into the chromosome, selected for by gentamicin resistance. Cells were then selected for a second recombination event involving the loss of the plasmid by aerobic growth on medium containing 7% sucrose. The resultant colonies were screened by PCR for a change in the size of the *rcc01081* locus amplicon and selected mutants were confirmed by sequencing using the primers 1081_seq_for and 1081_seq_rev. The *1932* mutant was generated in a similar fashion, using the primers 1932_up_for, 1932 up_rev, 1932_down_for, and 1932_down_rev. The *1932/ 1081* double mutant was generated by conjugating pZDJ 1081 into the *1932* strain and screening for mutants as described above (see Table S1 for primer sequences).

Complementation of the *rcc01081* and *rcc01932* mutations

The primers 1081_comp_up and 1081_comp_down, and 1932_comp_up and 1932_comp_down were used to amplify the entire *rcc01081* and *rcc01932* genes respectively. Amplicons were ligated into the multiple cloning site of the plasmid pIND4 (Ind *et al.*, 2009) as NcoI to HindIII fragments to produce the plasmids pIND1081, and pIND1932. These constructs allow for inducible expression of the *rcc01081* and *rcc01932* genes under control of an isopropyl β -D-1-thiogalactopyranoside (IPTG)-inducible *lac* promoter. The plasmid pIND1081 was then conjugated into *R. capsulatus* mutant strain *1081*, and the plasmid pIND1932 was conjugated into the strain *1932*, generating the strains *1081*[p1081] and *1932*[p1932]. For experiments using these complemented strains, the cultures were grown with 1 mM IPTG during growth to induce expression of each gene.

RcGTA recipient capability assay

A rifampicin-resistant RcGTA overproducer strain (Yen *et al.*, 1979), DE442, was used as a source of RcGTA for recipient capability and adsorption experiments. A sample of stationary phase culture grown phototrophically in YPS complex medium (Wall *et al.*, 1975), passed through a 0.2 μ m filter, was titred for RcGTA activity, and a diluted stock solution that produced ~ 800 rifampicin-resistant transductants per 100 μ l of donor filtrate (with stationary phase B10 cells as a recipient) was used for all experiments. All strains used as RcGTA recipients, other than 37b4, were derived from WT B10. Cells were grown aerobically in RCV defined medium with shaking at 200 r.p.m. to the stationary phase, harvested, and re-suspended in an equal volume of G-buffer [10 mM Tris-HCL (pH 7.8), 1 mM MgCl₂, 1 mM CaCl₂, 1 mM NaCl, 500 μ g ml⁻¹ BSA] (Solioz *et al.*, 1975). A transduction assay was then performed, where 100 μ l of RcGTA stock, 100 μ l of recipient cells, and 400 μ l of G-buffer are mixed together. This mixture was incubated for 90 min at 30°C with gentle agitation, after which 900 μ l of RCV medium were added, followed by incubation under the same conditions for 3 h. Cells were spread on RCV plates containing rifampicin and incubated aerobically for 3 days. Rifampicin-resistant colonies were counted and the average was corrected by subtracting the number of spontaneous rifampicin-resistant colonies (no addition of RcGTA, usually no more than 3%). Because of variability in total numbers of transductants between individual experiments, RcGTA recipient capability efficiencies are expressed as % WT (B10), with each experimental strain being compared with the WT control within the same experiment. In the case of the growth phase recipient capability experiment, total numbers of rifampicin-resistant colonies from a single representative experiment are shown. The statistical significance of results was evaluated by One-Way ANOVA. A *P*-value of 0.05 (95% confidence interval) was set as a cut-off for significance.

RcGTA adsorption assay

To quantitatively measure the ability of different strains to bind to RcGTA, cells were grown aerobically in RCV defined medium with shaking at 200 r.p.m. to the stationary phase, harvested, and re-suspended at the same density in G-buffer. The first step of a transduction assay was then performed, where 100 μ l of RcGTA solution, 100 μ l of recipient cells, and

400 μ l of G-buffer are mixed together. This mixture was incubated for 90 min at 30°C with gentle agitation, after which this mixture was passed through a 0.2 μ m filter. The residual levels of RcGTA in the filtrate (RcGTA that did not adsorb to cells) was quantified by performing another standard transduction assay using 100 μ l of this filtrate as the RcGTA donor, and WT *R. capsulatus* B10 cells as the recipient. As two separate controls, an assay that included no recipient cells for adsorption (no cells), and an assay that contained no RcGTA were also performed. For cell surface extract competitive inhibition assays, WT B10 cells were used as a RcGTA recipient strain in all cases. Because of variability in total numbers between experiments, adsorption efficiencies are expressed as a % of the value obtained with no cells, with each experimental strain being compared with the un-adsorbed control (with the exception of the growth curve experiment). The statistical significance of results was evaluated by One-Way ANOVA. A *P*-value of 0.05 (95% confidence interval) was set as a cut-off for significance.

Western blot

Filtrate from RcGTA adsorption assays was concentrated 10-fold in a SpeedVac concentrator (Thermo Electron Corp) and samples were boiled for 10 min in sample loading buffer (50 mM Tris-HCl pH 6.8, 2% SDS, 10% glycerol, 0.1% bromophenol blue, 1% β -mercaptoethanol) prior to gel loading. Samples were separated on a 12% SDS-PAGE gel and blotted on to a nitrocellulose membrane (Perkin-Elmer). The blotting was done using a Mini Trans-Blot apparatus (Bio-Rad) according to the manufacturer's specifications in electroblot buffer (27.5 mM Tris-Base, 192 mM glycine, 20% methanol) at 100 V constant voltage for ~ 1.5 h.

The primary antibody (rabbit) was raised against the *R. capsulatus* RcGTA capsid protein (Taylor, 2004). Primary antibody binding was detected using a peroxidase-linked anti-rabbit Ig secondary antibody (from donkey; Amersham) as part of the enhanced chemiluminescence (ECL) kit according to the manufacturer's instructions (Amersham).

Densitometry measurements of relative band intensity were performed using the program ImageJ (<http://rsbweb.nih.gov/ij/>).

Phenol-sulfuric acid carbohydrate quantification

Estimation of cell-associated carbohydrate (EPS) relative amounts was done using a modified phenol-sulfuric acid method (Liu *et al.*, 1973). *R. capsulatus* cells were grown phototrophically to the stationary phase in RCV defined medium, and the cell concentration normalized to 450 Klett units by the addition of medium. The cells from 1 ml of each normalized culture were harvested in a microcentrifuge at 16 500 *g* for 1 min (same centrifugation for all steps), and washed three times in 50 mM NaCl. Cells were re-suspended in 1 ml of 50 mM EDTA and incubated at 37°C for 30 min. Cells were then pelleted, and the resultant supernatant containing LPS and any tightly associated EPS, such as capsule, was subjected to a phenol-sulfuric acid colourimetric carbohydrate quantification in the following manner: 200 μ l of the sample and 200 μ l of 5% phenol were mixed together in a glass test tube; 1 ml of 93% sulfuric acid was added, and the tube agitated to ensure mixing. Colour was allowed to develop for 10 min. Colour intensity was then measured at

490 nm in a spectrophotometer, and compared with standards of known carbohydrate concentration. The sugar standards were a 50:50 mixture of sucrose and fructose diluted from a stock of known concentration. The statistical significance of results was evaluated by One-Way ANOVA. A *P*-value of 0.05 (95% confidence interval) was set as a cut-off for significance.

Capsule stain

Capsules were negatively stained using Anthony's capsule stain (Anthony, 1931). In brief, 1 ml of stationary phase *R. capsulatus* cultures was harvested by centrifugation, and re-suspended in 1 ml of carnation skim milk (skim milk powder was re-suspended in H₂O at 1% w/v). An inoculating loop of each re-suspended culture was spread onto a clean microscope slide and allowed to dry in the air. Smears were then stained with 1% crystal violet for 5 min, and washed gently and thoroughly with 20% copper sulphate. Slides were then allowed to dry in the air, and subsequently examined using oil-immersion phase contrast microscopy at 100× magnification.

Bioinformatic analyses

The predicted amino acid sequences for Rcc01081-1086, Rcc01932, and Rcc01958-1960 were used to perform a BLASTp search against the nr database (Altschul *et al.*, 1990). Selected sequences were globally aligned using the EMBOSS Needle program for pairwise alignments.

β-Galactosidase assays

The β-galactosidase specific activity of cells containing the *rcc01081::lacZ* promoter fusion was assayed as previously described (Leung *et al.*, 2012). Sonication was used to break cells, and total protein was measured using the Lowry method (Peterson, 1983), with bovine serum albumin as the standard. β-Galactosidase specific activities are presented as units per milligram of total protein.

Expression analysis of wild-type and gtaI mutant strains of *R. capsulatus*

Photoheterotrophic cultures for microarray analysis were grown in RCV defined medium at 30°C. Cells were harvested at the logarithmic and stationary phases of growth as determined by culture turbidity. RNA extraction, cDNA synthesis, target labelling and hybridization were all performed as previously described (Mercer *et al.*, 2010). Microarrays for a single sample of each strain in both phases of growth were performed by The Centre for Applied Genomics, The Hospital for Sick Children, Toronto, Canada. The raw data from the scanned arrays were normalized by Robust Multi-Array Analysis (RMA) (Irizarry *et al.*, 2003) in Affymetrix Expression Console v1.1 (Santa Clara, CA) and exported for analyses. The relationships between the gene features on the custom array and the sequenced genome (NCBI Accession Number NC_014034) have been previously described (Mercer *et al.*, 2010).

LPS silver stains

The LPS from *R. capsulatus* was visualized as previously described (Tsai and Frasch, 1982). LPS was purified by harvesting 1 ml of culture by centrifugation, followed by three washes in 1 ml of 50 mM NaCl. Cells were then re-suspended in 500 μ l of 20 mM EDTA and incubated for 30 min. Cells were then pelleted by centrifugation (14 500 r.p.m. for 1 min) and the supernatant (containing LPS) was removed, treated with proteinase K for 4 h at 50°C, and concentrated in a SpeedVac concentrator (Thermo Electron Corp). For LPS gels, material was loaded and electrophoresed as described in the *Western blot* section.

Surface polysaccharide extraction for inhibition of RcGTA binding

Surface polysaccharides (EPS/LPS) were purified in the following manner: 50 ml of each strain was grown aerobically in RCV medium with shaking at 200 r.p.m. until cultures reached the stationary phase. Cells were then harvested by centrifugation at 12 000 *g* for 30 min, followed by two consecutive washes in 50 mM NaCl. Cell pellets were then re-suspended in 10 ml of 50 mM EDTA, and incubated overnight at room temperature. Cells were then removed by centrifugation, and the resultant supernatant (containing EPS/LPS) was subsequently treated with proteinase K (1 mg ml⁻¹) for 4 h, followed by heating at 80°C for 1 h to deactivate the protease. Samples were then dialysed against dH₂O for 2 days with several buffer changes to remove EDTA, followed by quantification using the phenol-sulfuric acid method. Extracts were added to RcGTA adsorption reactions at a final concentration of 100 μ g ml⁻¹.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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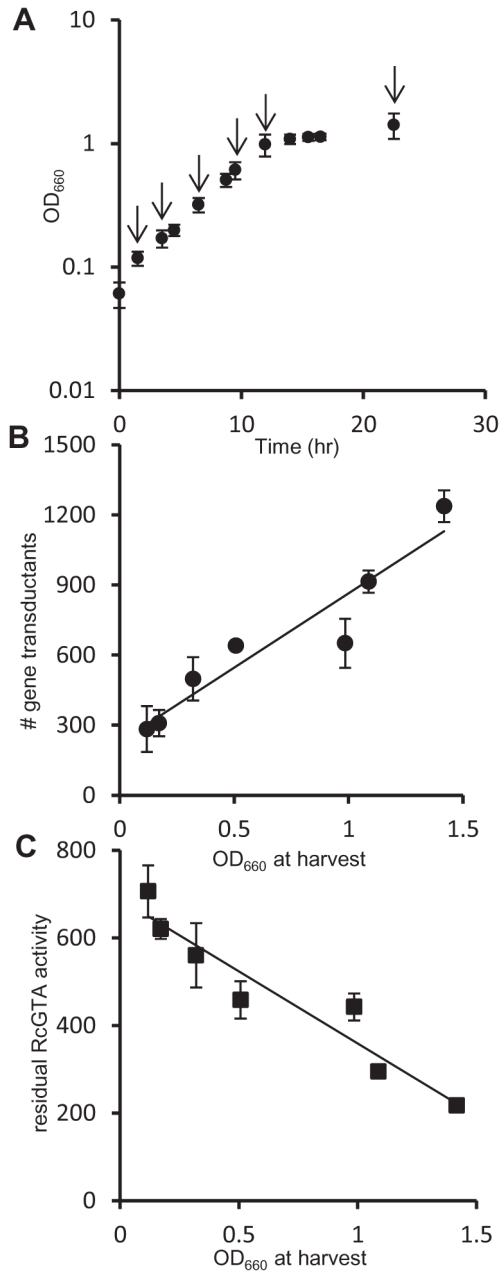


Fig. 1. Effect of growth phase on RcGTA recipient capability and adsorption

A. Growth curve of *R. capsulatus* B10 culture used for recipient capability and adsorption experiments. Arrows indicate time points where samples were taken for recipient capability and adsorption experiments. Error bars represent the variation in OD₆₆₀ between two duplicate cultures.

B. Number of rifampicin-resistant colonies obtained in a transduction assay using equal numbers of cells at different phases of growth as recipients for RcGTA. Error bars represent the variation between two samples (one sample from each culture).

C. RcGTA adsorption to cells at different growth phases. The vertical axis shows the number of rifampicin-resistant colonies derived from an adsorption assay (see *Experimental*

procedures), and the error bars represent the variation between two samples (one sample from each culture). A statistical correlation analysis of the data in Fig. 1B and C is given in Table S2.

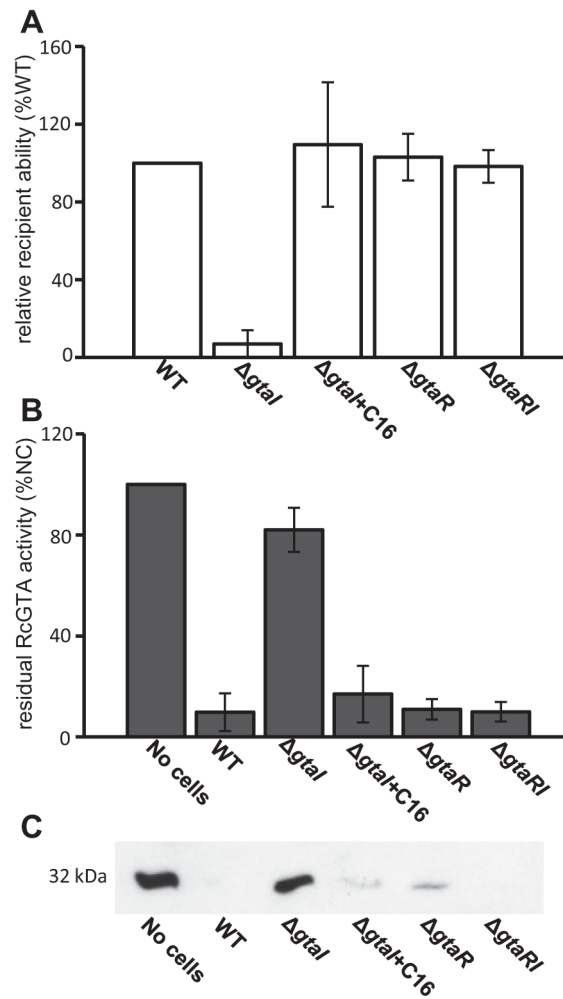


Fig. 2. Comparison of RcGTA recipient capability of QS mutant strains with their ability to adsorb RcGTA particles

A. Ability of WT, *gtal*, *gtar*, *gtarI*, and *gtal* supplemented with C16-acyl-HSL strains to be transduced by RcGTA containing a Rifampicin resistance marker.

B. RcGTA adsorption ability of strains (values are presented as a percentage of the number of Rif^R transductants obtained in the unabsorbed control where no cells were added to the reaction mixture, indicated by %NC).

C. Western blot of adsorption assay filtrate probed with RcGTA capsid protein antiserum.

Error bars represent the standard deviation between samples ($n = 3$). Statistical analysis was done by One-Way ANOVA, given in Tables S3 and S4.

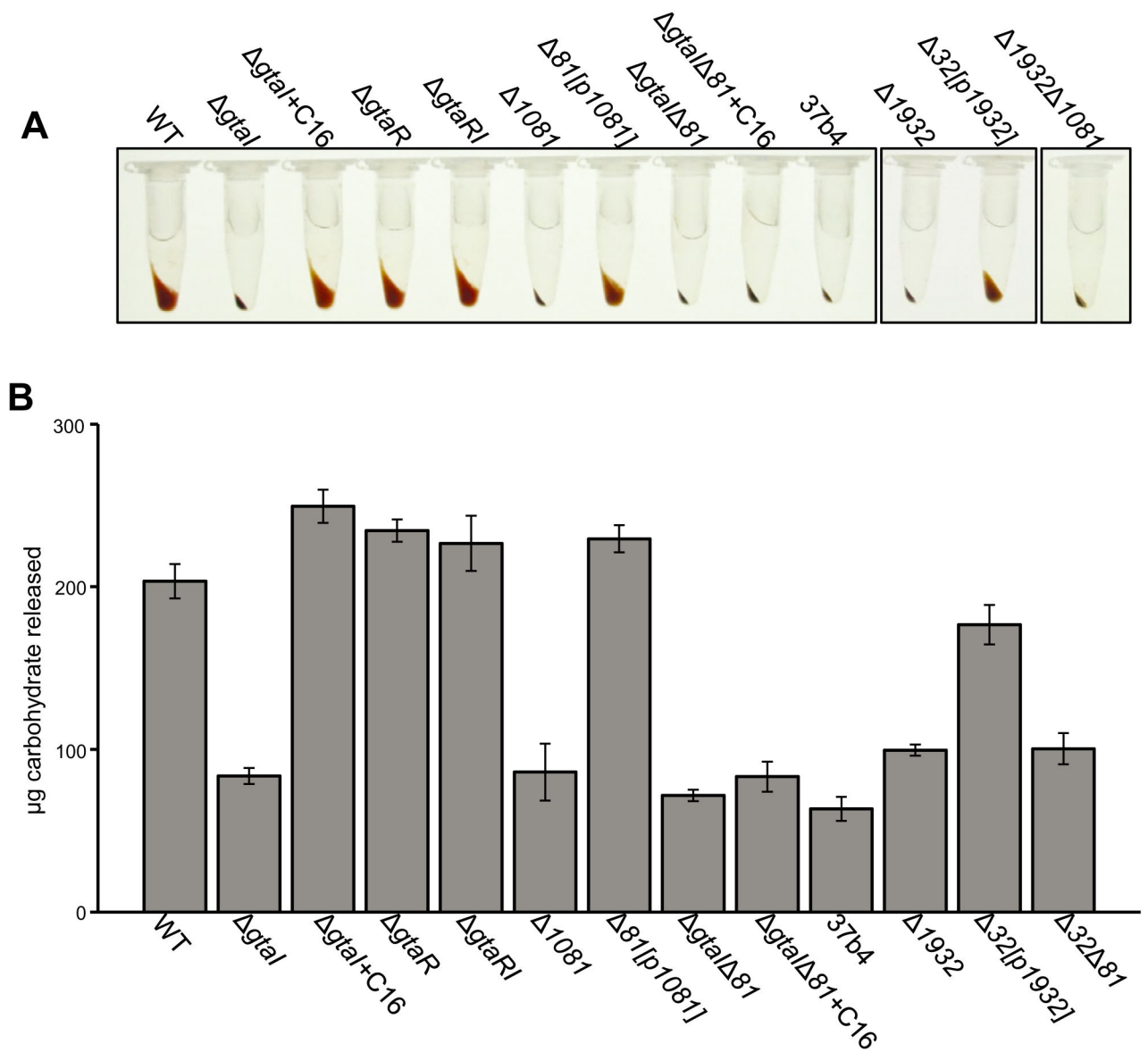


Fig. 3. Evaluation of EPS production in *R. capsulatus* QS and EPS biosynthesis mutants

A. Pellet formation of equal numbers of cells of phototrophically grown B10 (WT), *gtaI*, *gtaI* + C16-acyl-HSL, *gtaR*, *gtaRI*, *1081*, *1081*[p1081], *gtaI 1081*, *gtaI/ 1081* + C16-acyl-HSL, *1932*, *1932*[p1932], *1932/ 1081* and WT 37b4 *R. capsulatus* strains after centrifugation for 1 min.

B. Levels of cell-associated EPS production in an extract of each strain as measured by a phenol-sulfuric acid assay. Error bars represent the standard deviation between samples ($n = 3$), values were calculated based on standard samples with a known amount of a 1:1 mixture of glucose and fructose. Statistical analysis was done by One-Way ANOVA, given in Table S5.

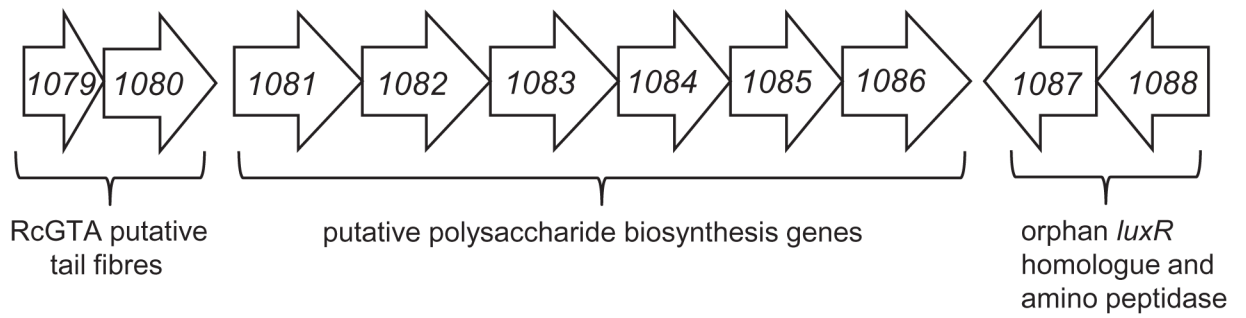


Fig. 4. Genomic organization of the *rcc01081-1086* gene cluster, and surrounding genes (not to scale). Arrows indicate the direction of transcription. Immediately upstream of *rcc01081-1086* are two predicted bacteriophage tail fibre genes, *rcc01079* and *1080*, and downstream is a putative amino peptidase, *rcc01087*, and a LuxR homologue, *rcc01088*.

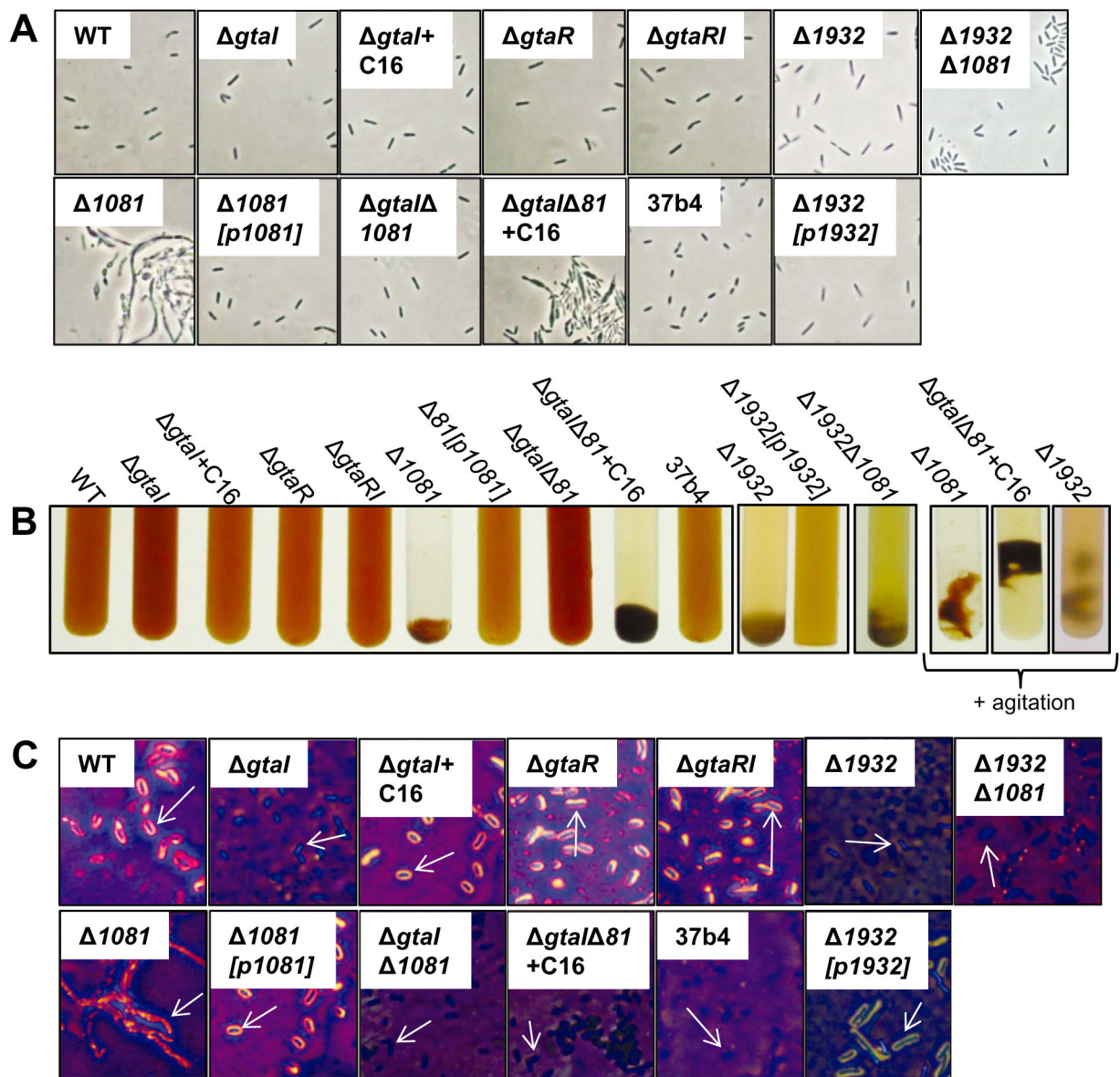


Fig. 5.

Phase contrast microscopy, capsule stain images, and culture macroscopic morphology of WT B10 (WT), *gtaI*, *gtaI* + C16-acyl-HSL, *gtaR*, *gtaRI*, *1081*, *1081*[p1081], *gtaI/1081*, *gtaI/1081* + C16-acyl-HSL, *1932*, *1932*[p1932], *1932/1081* and WT 37b4 *R. capsulatus* strains.

A. Phase contrast microscopy images of the strains listed above, at 100 \times magnification.

B. Images of tubes of phototrophically grown cultures. On the far right, images of agitated cultures of *1081*, *gtaI/1081*, and *1932* are shown.

C. Capsule stain images of WT, QS and EPS biosynthesis mutant strains.

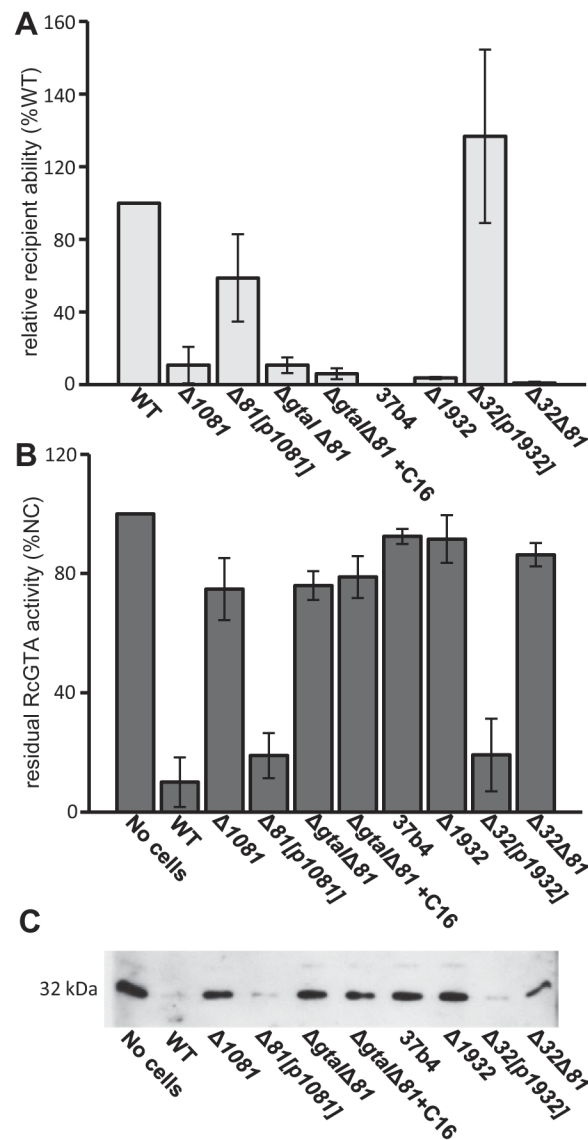


Fig. 6. Comparison of WT B10 (WT), 1081, 1081[p1081], gtaI 1081, gtaI/ 1081 + C16-acyl-HSL, 1932, 1932[p1932], 1932/ 1081 and 37b4 WT strains recipient capability and ability to adsorb RcGTA particles

A. Ability of strains to be transduced by RcGTA as measured by the number of rifampicin-resistant colonies arising in a transduction assay.

B. Adsorption ability of strains as measured by residual RcGTA activity in culture filtrate (values are presented as a percentage of the number of Rif^R transductants obtained in the unabsorbed control where no cells were added to the reaction mixture, indicated by %NC).

C. Western blot adsorption assay of a residual RcGTA sample, probed with RcGTA capsid protein antiserum. Error bars represent the standard deviation between samples ($n = 3$).

Statistical analysis was done by One-Way ANOVA, given in Tables S3 and S4.

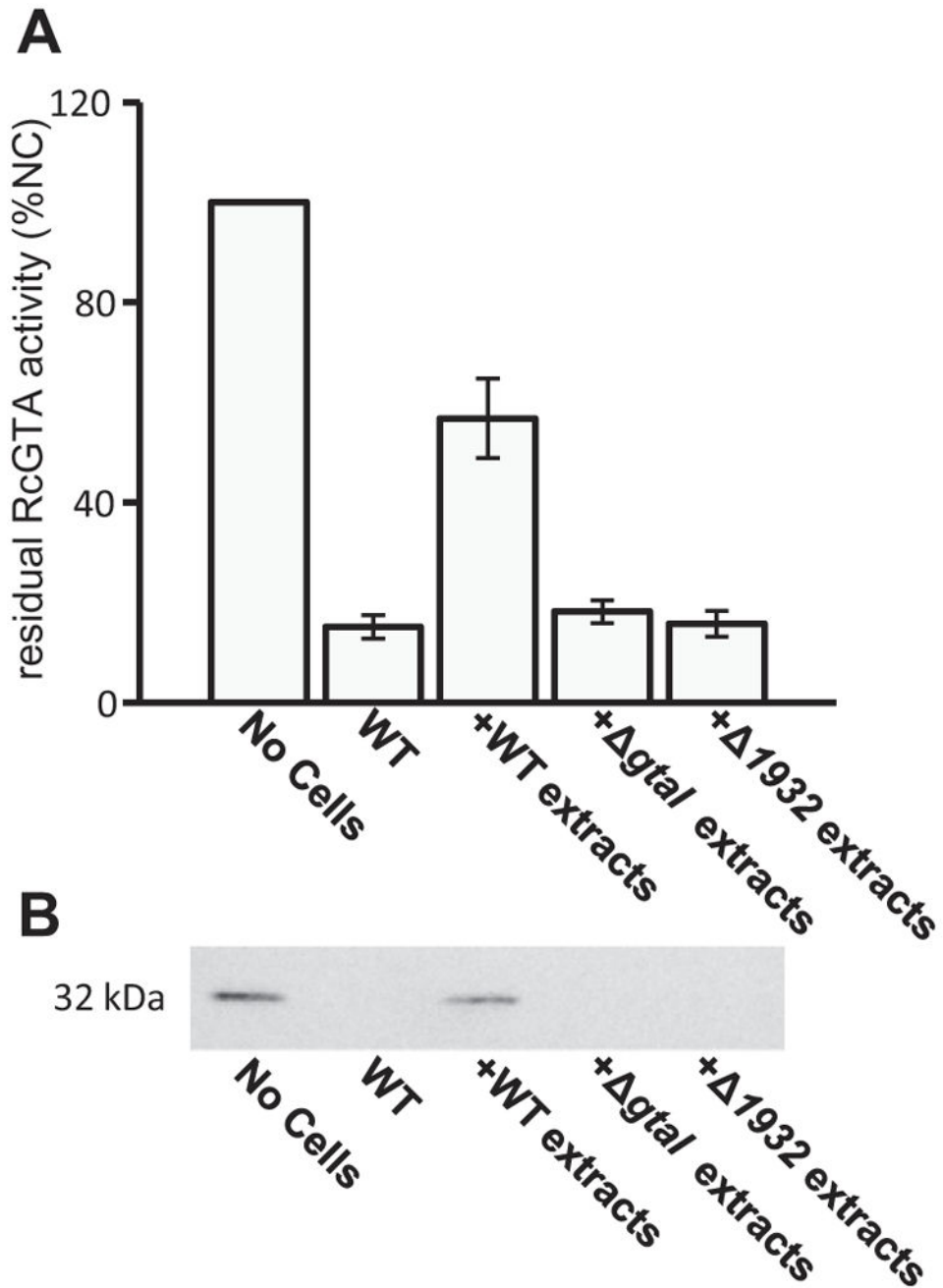


Fig. 7. Inhibition of RcGTA adsorption by addition of cell surface extracts

A. Relative amount of transduction-proficient RcGTA present after an adsorption assay, with cell surface extracts added to the reaction mixture where indicated; No Cells denotes a control, in which RcGTA was added directly to recipient cells; WT indicates the addition of WT B10 cells to RcGTA before removal of cells by filtration and use of the RcGTA-containing filtrate for gene transduction; +WT extracts denotes the addition of cell surface extracts of WT B10 cells to RcGTA before removal of cells by filtration and use of the RcGTA-containing filtrate for transduction; + *gal* extracts, and + *1932* extracts denote adsorption assays where cell surface extracts from each indicated strain were added to the

transduction mixture (containing WT B10 cells). Adsorption values were measured by the amount of residual RcGTA in the filtrate (values are presented as a percentage of the number of Rif^R transductants obtained in the control where no cells were added to the reaction mixture).

B. Western blot of cells treated as described in (A), and probed with capsid antiserum. Statistical analysis was done by One-Way ANOVA, given in Table S7.

Table 1

Relative transcription of putative EPS/capsular polysaccharide gene cluster and additional required genes as analysed by microarray analysis.

Locus	Genome annotation	WT/ <i>gtaI</i> log ^a	WT/ <i>gtaI</i> stat ^b	stat/log ^c
<i>rcc01081</i>	Glycosyl transferase, group 1	4.53	3.06	2.02
<i>rcc01082</i>	Glycosyl transferase, group 1	4.02	2.83	1.49
<i>rcc01083</i>	UDP-glucuronate 5'-epimerase (UDP-glucuronic acid epimerase)	2.59	2.16	1.51
<i>rcc01084</i>	Membrane protein, putative	2.94	2.12	1.44
<i>rcc01085</i>	Hypothetical protein	3.73	2.36	1.48
<i>rcc01086</i>	Glycosyl transferase, group 1	2.65	2.92	1.5
<i>rcc01932</i>	Glycosyl transferase, family 4	8.93	5.36	3.45
<i>rcc01958</i>	Tyrosine-protein kinase etk	1.66	1.39	1.57
<i>rcc01959</i>	Low molecular weight protein-tyrosine-phosphatase etp	2.08	1.27	1.8
<i>rcc01960</i>	Polysaccharide export protein	2.67	1.75	1.01

^aRatio of WT to *gtaI* microarray values for cells grown to the exponential phase of growth.

^bRatio of WT to *gtaI* microarray values for cells grown to the stationary phase of growth.

^cRatio of microarray values of cells in the stationary phase to cells in the exponential phase.

Values are expressed as the fold change in a *gtaI* mutant relative to wild type, and log versus stationary phase in the wild type. All cultures grown in RCV defined medium.

Table 2

Bioinformatic analysis of putative EPS/capsular polysaccharide biosynthesis gene cluster and additional related genes.

Locus	Genome annotation	Best experimentally verified BLASTp hit	% ID	# Predicted TMS	Predicted function
<i>rcc01081</i>	Glycosyl transferase, group 1	PglA; <i>Helicobacter pullorum</i>	28.7	0	Repeat unit assembly
<i>rcc01082</i>	Glycosyl transferase, group 1	WfV; <i>Shigella dysenteriae</i>	39	1	Repeat unit assembly
<i>rcc01083</i>	UDP-glucuronate 5'-epimerase	Gla; <i>Cronobacter malonaticus</i>	50.4	0	Repeat unit monosaccharide biosynthesis
<i>rcc01084</i>	Membrane protein, putative	Wzx; <i>Cronobacter malonaticus</i>	21.3	8	Repeat unit flippase
<i>rcc01085</i>	Hypothetical protein	Wzy; <i>Escherichia coli</i>	22.8	9	Polysaccharide polymerase
<i>rcc01086</i>	Glycosyl transferase, group 1	WbpR; <i>Pseudomonas aeruginosa</i>	17.5	0	Repeat unit assembly
<i>rcc01092</i>	Glycosyl transferase, family 4	WecA; <i>Ralstonia solnacearum</i>	33.7	9	Repeat unit chain initiation
<i>rcc01098</i>	Tyrosine-protein kinase etk	Wzc; <i>Vibrio vulnificus</i>	38	2	Polysaccharide export
<i>rcc01099</i>	Low molecular weight protein-tyrosine-phosphatase etp	Wzb; <i>Vibrio vulnificus</i>	41.1	1	Polysaccharide export
<i>rcc01060</i>	Polysaccharide export protein	Wza; <i>Vibrio vulnificus</i>	33.4	0	Polysaccharide export

Data shown include best BLASTp hits, pairwise alignment identities, number of predicted transmembrane segments (TMS) as indicated by a hydropathicity plot (window size = 19), and predicted function in EPS/CPS biosynthesis.