INTRODUCTION

Several members of the Alphaproteobacteria produce gene transfer agents (GTAs), small bacteriophage-like particles that package random segments of cellular DNA and mediate horizontal gene transfer. Homologous GTA genes are located in a widely conserved gene cluster in bacterial chromosomes, and the cluster appears to have established itself early in the evolutionary history of the Alphaproteobacteria with subsequent vertical inheritance [1, 2]. The model GTA is RcGTA, produced by the purple non-sulfur bacterium Rhodobacter capsulatus, a metabolically versatile organism commonly found in nutrient-rich aquatic environments [3–6]. In laboratory culture a small subset of the population produces RcGTA, and both production and recipient capability (the capability of cells to receive RcGTA-borne DNA) are strain-dependent [7–9]. Efficient gene transfer involves binding to a polysaccharide capsule receptor by spikes located on the RcGTA capsid, and several natural competence-like (com) genes that are required for cells to receive DNA [10–13]. Strikingly, both RcGTA particle production and expression of the natural transformation-like genes are regulated by the same cellular regulators, including the conserved CckA-ChpT-CtrA phosphorelay (reviewed in [14]).

Both RcGTA production and recipient capability are growth phase-regulated: RcGTA gene cluster transcription, protein production and transduction frequencies from donor cells greatly increase as cultures transition into the stationary phase [15–17]. Similarly, production of the cell capsule receptor and the recipient capability of cells increase as laboratory cultures become more dense [12]. A quorum sensing system composed of the N-acyl-homoserine lactone-synthesizing GtaI and sensor GtaR regulates both RcGTA production and recipient capability [18, 19]. However, an increase in RcGTA transcription between the early exponential and stationary phases was reported to be independent of GtaI [19], indicating that there are additional growth phase-dependent signals stimulating RcGTA production at the transition into the stationary phase.

Here, we investigated whether RcGTA production is stimulated by nutrient depletion. Cells depleted of carbon greatly stimulated RcGTA production and release, with little or no effect observed from depletion of nitrogen, phosphorus or light (the source of ATP). Blockage of amino acid biosynthesis, either by mutation or treatment of wild-type (WT) cultures with inhibitors, similarly stimulated RcGTA production. Therefore, R. capsulatus upregulates RcGTA production in response to signals arising from nutrient depletion, particularly amino acid depletion. Additionally, the sole RelA-SpoT homologue (RSH) in R. capsulatus and the RNA polymerase omega (ω) subunit were required for maximal or appreciable RcGTA production, respectively.
METHODS

Growth conditions and nutrient depletion

Strains are listed in Table S1 (available in the online Supplementary Material). For general growth of the genome-sequenced, rifampin-resistant \textit{R. capsulatus} strains SB1003 (RcGTA WT) and DE442 (RcGTA overproducer) \cite{20,22}, cells were cultivated aerobically in RCV minimal medium \cite{23}. For experiments, cultures were inoculated to an optical density at 660 nm of 0.15 (approximately $7.2 \times 10^7$ c.f.u. ml$^{-1}$) in the minimal medium RCV and incubated phototrophically (anaerobically with illumination) at 30°C in 16.5 ml screw-cap glass tubes, unless otherwise stated. For nutrient depletion experiments, cultures were grown to the late exponential phase (250 Klett units), cells pelleted by centrifugation (3500 rcf) and suspended in RCV medium lacking (depleted) or containing (replete) the specific nutrient, and incubated for 2 h before the nutrient was added back to depleted cultures. For experiments involving amino acid auxotrophs, RCV medium was supplemented with 0.2 mM histidine or 4 mM serine. The histidine biosynthesis inhibitor 3-amino-1,2,4-triazole (3AT) was dissolved to 1 M in dimethyl sulfoxide (DMSO). Cells were grown to 250 (for transduction and western blot) or 150 (promoter activity) Klett units in RCV medium and treated with either DMSO (blunted using T4 DNA polymerase) or 150 (promoter activity) µM 3-aminotriazole (3AT) or 10 mM 3AT and 0.1 mM histidine.

\textit{E. coli} was cultivated in LB medium \cite{24} supplemented with 0.2 mM ampicillin (100 µg ml$^{-1}$), tetracycline HCl (10 µg ml$^{-1}$), gentamicin sulfate (50 µg ml$^{-1}$) or kanamycin sulfate (3 µg ml$^{-1}$), as appropriate.

\textit{Escherichia coli} strain DH5α \cite{24} lambda pil was used for general cloning work. For conjugation of plasmids to \textit{R. capsulatus}, \textit{E. coli} S17-1 \cite{25} lambda pil or TEC5 \cite{26} was used. \textit{E. coli} was cultivated in LB medium \cite{24} supplemented with either ampicillin (100 µg ml$^{-1}$), tetracycline HCl (10 µg ml$^{-1}$), kanamycin sulfate (50 µg ml$^{-1}$) or gentamicin sulfate (3 µg ml$^{-1}$), as appropriate.

Construction of plasmids and targeted mutations

Plasmids are listed in Table S2. The histidine auxotrophic strain \textit{hisB} :: \textit{KIXX} was created by amplifying \textit{hisB} (rcc03183) using atattgtagctgccgacacgccgcaac and atatgctggtgagagccgagaag, and cloned into pUC19 \cite{24} using PstI and SacI. The pUC4-KIXX \cite{27} Smal fragment (confering kanamycin resistance) was introduced into the XhoI site (blunted using T4 DNA polymerase) in forward orientation to construct pHisBKIXX, disrupting the 5′ half of \textit{hisB}. The \textit{hisB} :: \textit{KIXX} mutant was created by RcGTA-transduction into SB1003 using TEC5 and DE442 \cite{28}. The markerless serine auxotrophic strain Δ\textit{serB} was created by amplifying \textit{serB} (rcc03445) upstream and downstream fragments using primer pairs atattgtagctgccgacacgccgcaac and atattgtagctgccgagagccgagaag, and atatgctggtgagagccgagaag and atatgctggtgagagccgagaag. The ampiclons were joined using Bsal (underlined) and cloned into the SacI (\textbf{bold}) and Sall (\textit{italicized}) sites of the suicide plasmid pZJD29A, which encodes gentamicin resistance, and the \textit{saCB} counter-selection marker (Z. Jiang and C. E. Bauer, personal communication), yielding plasmid pZKOSerB. The Δ\textit{serB} mutant was created by conjugating pZKOSerB into WT strain SB1003 and selecting for primary (gentamicin resistance) and secondary (sucrose resistance) crossovers.

A strain lacking the RelA SpoT homologue (RSH) was created by transduction of the tetracycline resistance-disrupted \textit{spoT} fragment to SB1003 from the hvrA \textit{spoT} double-mutant strain SM05 \cite{29}. The loss of \textit{spoT} was confirmed by PCR amplification of \textit{spoT} (Fig. S2a). The \textit{spoT} complementation plasmid pSspoT was created by amplifying the coding region of \textit{spoT} (rcc03317) using primers atatgctggtgagagccgagaag and atatgctggtgagagccgagaag and cloned into pLND4 \cite{30} 3′ of the lac promoter \textit{P}_{\text{Al}}1004/03 using Ncol and BamH1. The \textit{rpoZ} :: \textit{KIXX} mutant and complementation plasmid pCrpoZ were constructed by amplifying \textit{rpoZ} (rcc03318) using atatgctggtgagagccgagaag and atatgctggtgagagccgagaag and cloned into plasmid pCM62 using PstI and SacI sites to create pCrpoZ. The KIXX Smal fragment was cloned into the XmnI site of pCrpoZ in forward orientation to construct pCrpoZKIXX, disrupting the 16th codon of \textit{rpoZ}, and the \textit{rpoZ} :: \textit{KIXX} mutants were constructed by transduction from DE442(pCrpoZKIXX).

GTA transduction

GTA transduction assays were performed by measuring the transfer of rifampin resistance from filtered (0.2 µm pore size) culture supernatant to the rifampin-sensitive \textit{R. capsulatus} WT strain B10, as previously described \cite{13,31}.

Western blots

Western blots to detect the RcGTA capsid protein were performed using capsid protein anti-serum as previously described \cite{7,31}. The approximate migration of molecular markers is indicated.

Promoter activity assays

The RcGTA gene cluster promoter activity was performed by measuring the $\beta$-galactosidase activity in cells containing the promoter-\textit{lacZ} reporter p601-g65 \cite{18}, as previously described \cite{31}.

Bioinformatic analyses

DNA and protein sequences were routinely inspected and analysed using Artemis (http://www.sanger.ac.uk/science/tools/artemis, \cite{32}), BLAST (http://blast.ncbi.nlm.nih.gov, \cite{33}), PFAM (http://pfam.xfam.org, \cite{34}) and SMART (http://smart.embl-heidelberg.de, \cite{35}). In silico assembly of
DNA sequences was performed using Serial Cloner (http://serialbasics.free.fr/Serial_Cloner). Global amino acid sequence alignment was performed using EMBOSS NeedleP (http://www.ebi.ac.uk/Tools/psa/emboss_needle, [36]).

**Statistical analysis**

The statistical significance of results was evaluated by the t-test or by ANOVA followed by Tukey’s multiple comparisons test (GraphPad Prism 7). Significance (0.05 set as a cut-off) is indicated with an asterisk (*) in Figures.

**RESULTS**

**Carbon depletion greatly stimulates RcGTA production and transduction**

The stationary phase of bacterial growth in laboratory culture is typically a result of depletion of nutrients and/or a build-up of toxic byproducts. Earlier research indicated that the composition of the growth medium influences RcGTA production [37], and we subsequently reported that the release of RcGTA from cells is stimulated in media low in inorganic phosphate concentration [31]. To investigate whether depletion of a specific nutrient induced RcGTA production, we cultivated *R. capsulatus* in the minimal medium RCV and transiently incubated cells in media depleted of the nutrients carbon, nitrogen or phosphorus (malic acid, ammonium sulfate or potassium phosphate, respectively). The amount of the RcGTA capsid protein present in the growth medium (supernatant) was greatly increased after carbon depletion (Fig. 1a; see Fig. S1 for biological replicate and loading controls), and gene transduction increased 21-fold (Fig. 1b) compared to a carbon-replete control. Carbon depletion stimulated RcGTA production (and not only the release), as the total amount of RcGTA capsid protein (intra- and extracellular) was greatly increased by the treatment compared to the control (Fig. 1a).

Compared to carbon depletion, smaller and less reproducible increases in the amount of extracellular capsid protein (Figs 1a and S1) were observed after nitrogen and phosphate depletion, and we therefore focused our attention on carbon depletion. Photoheterotrophic cultures of *R. capsulatus* utilize carbon for biosynthesis rather than energy generation [38], and little or no increase in RcGTA capsid protein was detected after depletion of light energy (Fig. 1a).

Therefore, biosynthetic carbon depletion, not energy depletion, is the main stimulus of RcGTA production in these malic acid-depleted cultures.

**RcGTA production is stimulated by amino acid depletion**

Carbon is a major nutrient and is involved in several anabolic pathways in the cell, including amino acid biosynthesis. We tested whether RcGTA production was stimulated after amino acid depletion by constructing targeted mutations in histidine and serine biosynthesis pathways. The SB1003-derived *hisB::KIXX* mutant, lacking the histidine biosynthesis gene *hisB* (*rcc01183*, encoding imidazoleglycerol-phosphate dehydratase, EC 4.2.1.19), was unable to grow on the minimal medium RCV unless supplemented with exogenous histidine, indicating that the strain is auxotrophic for histidine (Fig. S2d). Temporary histidine depletion increased transduction frequencies 8.2-fold for the *hisB* mutant relative to a histidine-replete control (Fig. 2a). In contrast, no increase was observed for the prototrophic parental strain SB1003 after depletion (0.76 of histidine-replete frequency). Similarly, histidine depletion increased the amounts of the RcGTA capsid protein in the culture supernatant and cell pellet fractions of the *hisB* mutant (Fig. 2b). Addition of the histidine biosynthesis inhibitor 3AT to WT SB1003 cultures similarly increased transduction frequency 8.9-fold compared to the control (Fig. 2c), and increased the levels of RcGTA capsid protein in both cell pellet and supernatant fractions (Fig. 2d). The effect of 3AT on RcGTA production was specific for histidine, as the effect of 3AT on transduction was offset by the addition of exogenous histidine (1.2-fold of control, Fig. 2c). 3AT treatment of cells increased RcGTA production by stimulating transcription of RcGTA genes.

The serine biosynthesis pathway was disrupted by deleting the *serB* (*rcc03445*, phosphoserine phosphatase, EC 3.1.3.3)
coding region in strain SB1003. This ΔserB mutant required exogenous serine for growth (not shown), indicating that it was unable to synthesize serine and the serine-derived amino acids glycine and cysteine. Temporary depletion of serine increased transduction 13.5-fold in ΔserB mutant cultures compared to serine-replete controls (Fig. 2f). Because RcGTA production is stimulated after depletion of both histidine and serine, it appears that amino acid depletion in general stimulates RcGTA production.

### The sole RelA-SpoT homologue and the RNA polymerase omega subunit are involved in RcGTA production

Amino acid starvation in bacteria typically triggers the ppGpp-mediated stringent response, resulting in major changes in the gene expression profile of cells [39–41]. ppGpp interacts with the relatively poorly studied RNA polymerase omega (ω) subunit that has for a long time been implicated in the stringent response in *E. coli* [42–45]. As in most bacteria [46], *R. capsulatus* encodes a single, multi-domain RelA SpoT homologue (RSH), historically referred to as SpoT [29]. As in *E. coli*, *R. capsulatus* ω is encoded next to spoT (Fig. 3a) and contains the N-terminal MAR motif (Fig. 3b) thought to be essential for ppGpp binding to RNAP ω [47]. We therefore investigated the involvement of the ppGpp-associated proteins SpoT and ω in RcGTA production.

Masuda et al. [29] showed that *R. capsulatus* lacking spoT failed to produce ppGpp after inhibition of tRNA^ser^ aminoacylation [29], indicating that starvation of a single amino acids triggers ppGpp production in *R. capsulatus*. Loss of spoT produces a pigmentation defect ([29], Figs 3c and

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**Fig. 2.** Amino acid depletion stimulates RcGTA production (a) Ratio of transduction frequencies for WT strain SB1003 and SB003 hisB mutant strains temporarily depleted of exogenous histidine to replete samples. (b) Western blot of SB1003 hisB mutant culture supernatant and cell pellet fraction. Biological replicates were temporary depleted of exogenous histidine as indicated. (c) Transduction frequencies of SB1003 cultures treated with DMSO (control; the 3AT solvent), the histidine biosynthesis inhibitor 3AT or 3AT and exogenous histidine. (d) Western blot of SB1003 culture supernatant and cell pellet fraction after DMSO (control) or 3AT treatment. (e) RcGTA promoter (β-galactosidase) activity for SB1003 containing the lacZ reporter plasmid p60-g65 after treatment with DMSO (control) or 3AT (Fig. 3a). (f) Transduction frequencies of SB1003 ΔserB cultures non-depleted (control) and temporarily depleted of exogenous serine. Blots (b and d) were probed with RcGTA capsid anti-serum. Bars indicate mean values and error bars the standard deviation of at least three biological replicates (a, c, e and f). A star (*) indicates a statistically significant difference (a, c, e and f: t-test, P-value<0.05; c: ANOVA (F(df,6)=13.27; P=0.0063) followed by Tukey’s test, alpha=0.05).
S2b), which we found was restored by expressing spoT in trans (Fig. 3c).

Cells lacking spoT grew well (Fig. S2c), but produced reduced levels of RcGTA: the mutant produced low levels of RcGTA capsid protein (Fig. 3d) and transduction was decreased to 22% of the level obtained from the parental strain (Fig. 3e). Capsid production was restored by introducing spoT in trans (Fig. 3c, d), indicating that the reduced RcGTA production was due to the loss of the sole RSH-encoding gene. It therefore appears possible that nutrient depletion may stimulate RcGTA production by stimulating ppGpp synthesis by RSH.

In E. coli, ppGpp is known to bind the RNAP ω subunit and modulate gene expression [42–45]. To investigate whether the R. capsulatus ω protein is involved in RcGTA production, we constructed a strain containing an rpoZ (encoding RCAP01160) deletion (ΔspoT), vector expressing spoT in trans (SpoT) or empty control vector pIND4 is indicated (c and d). For (e), the transduction frequencies (average of two technical replicates) were determined for three biological replicates, and bars give mean values and error bars the standard deviation. A star (*) indicates a statistically significant difference (t-test, P-value<0.05).

### Fig. 3. The sole RSH homologue spoT is required for maximal RcGTA production. (a) Genetic context of the spoT and rpoZ (encoding the RNAP ω subunit) genes. The 5’-most gene folK encodes an enzyme in folate biosynthesis and the 3’-most rcc03316 encodes a predicted transmembrane protein of unknown function. The fragment deleted in spoT (ΔspoT) and the KIXX-disruption site in rpoZ are indicated. Drawn to approximate scale. (b) The N-terminal amino acid sequence of R. capsulatus and E. coli ω subunit. The MAR motif, thought to be essential for ppGpp binding, is highlighted. (c) Colony pigmentation of cells. (d) Western blot of cell pellet and culture supernatant fraction, probed using RcGTA capsid protein antiserum. (e) Transduction frequencies from culture supernatant. Presence of the spoT mutation (ΔspoT), vector expressing spoT in trans (SpoT) or empty control vector pIND4 is indicated (c and d). For (e), the transduction frequencies (average of two technical replicates) were determined for three biological replicates, and bars give mean values and error bars the standard deviation. A star (*) indicates a statistically significant difference (t-test, P-value<0.05).
Loss of ω resulted in a marked diminution in the amount of RcGTA capsid protein (Fig. 4a), an effect that was similar or identical to the loss of the response regulator CtrA, the enigmatic gene rcc01865 or the LexA repressor [48–50].

To investigate whether the effect of ω was specific for the ~15 kb RcGTA gene cluster (encoding the capsid protein and most structural RcGTA proteins), we investigated whether the loss of ω blocked cell lysis. The lytic release of RcGTA is mediated by an endolysin-holin system that is encoded distantly from the RcGTA gene cluster, but regulated by CtrA [7, 8, 31]. Loss of ω abolished detectable lysis (Fig. 4b), indicating that ω is required for production of most, if not all, RcGTA proteins. Because introduction of rpoZ in trans restored both capsid production and cell lysis (Fig. 4a, b), the disruption was specific for rpoZ and the effects on RcGTA were not due to polar effects on the 3’ spoT gene (Fig. 3a).

To summarize, production of the R. capsulatus gene transfer agent was found to be stimulated by depletion of carbon or amino acids. Furthermore, the RSH and ω proteins are required for maximal or appreciable RcGTA production, respectively, indicating that the effect of nutrient depletion may be mediated through ppGpp and the RNAP ω subunit.

DISCUSSION

Gene transfer was markedly increased after cells were depleted of either organic carbon or amino acids (Figs 1 and 2) due to an increase in RcGTA production and subsequent release. This indicate that horizontal gene transfer among R. capsulatus cells is stimulated by nutrient signals, and possibly by a nutrient stress response. We suspect that depletion of multiple amino acids acts additively or synergistically on RcGTA production, as cells unable to produce serine and the serine-derived amino acids glycine and cysteine had increased RcGTA production (14-fold of control) compared to cells unable to produce histidine (8-fold) after depletion of exogenous serine or histidine, respectively. Furthermore, carbon depletion, which presumably results in a depletion of multiple amino acids, increased transduction at even greater levels (21-fold).

RcGTA is released from cells by regulated cell lysis [31], and it appears that carbon depletion may have a stronger effect than amino acid depletion on the release of RcGTA: following amino acid depletion, cells were observed to have increased capsid levels both intracellular and extracellularly (Fig. 2b). In contrast, carbon-depleted cells had intracellular capsid levels similar to the control, but greatly increased extracellular levels (Fig. 1a), which may be the result of an increased proportion of RcGTA-producing cells releasing particles by lysis.

Horizontal gene transfer in other organisms has also been reported to be influenced by nutrients. Several competence systems of naturally transformable bacteria are induced by environmental factors [51]. Like the production of RcGTA and development of RcGTA recipient capability [12, 15, 17], natural competence in Haemophilus influenzae and Pseudomonas species is growth phase dependent and maximal in the stationary phase [52–54]. Furthermore, H. influenzae competence development is greatly stimulated by transfer of cells from a complex, rich medium to a starvation (minimal) medium, and is regulated by carbon catabolite repression that requires cyclic AMP (cAMP) production and the transcriptional regulator CRP (cAMP-binding protein) [55–57]. Catabolite repression was also found to be an important regulator of chitin-dependent competence in Vibrio cholera [58]. In contrast, the competence of Acinetobacter species is maximal in the exponential phase and appears to be stimulated by a ‘nutrient upshift’ after dilution of cultures [59, 60].

Several nutrient stresses have been reported to stimulate a ppGpp-mediated stringent response [39, 40]. Our mutant

![Fig. 4](image-url)
strain ΔspoT lacking RSH, the only known ppGpp-producing enzyme in *R. capsulatus* [29], produced low levels of RcGTA (Fig. 3d, e), and it was previously shown that *R. capsulatus* induces ppGpp production in response to depletion of a single amino acid [29] in contrast to some other members of the Alphaproteobacteria [61]. We also found that the *R. capsulatus* RNAP subunit ω, which forms one of two binding sites for ppGpp on RNAP [44, 45, 62] and has long known to be involved in ppGpp-mediated effects in *E. coli* [42, 43], is required for detectable RcGTA capsid protein production and cell lysis (Fig. 3e, f).

It is tempting to speculate that amino acid depletion stimulates RcGTA production by triggering the ppGpp-mediated stringent response [39], but additional work would be needed to assign a proven connection between a classical stringent response and induction of RcGTA-mediated gene transfer. We also note that light depletion did not appear to stimulate RcGTA production (Fig. 1a), whereas it was reported that the related organism *R. sphaeroides* induces ppGpp production in response to light depletion [63]. Nevertheless the data in this paper clearly show that depletion of amino acids needed for biosynthetic purposes induces transcription of RcGTA structural genes, the release of RcGTA particles to the extracellular milieu and major increases in gene transfer to recipient cells.

The involvement of the RNAP ω subunit in RcGTA production may be specific (for example, through involvement in a putative stringent response) or due to a more general effect, as ω has been reported to be important for the assembly and stability of RNAP in both Gram-negative and -positive bacteria [64–67]. Richard et al. [68] reported that the *R. capsulatus* ω subunit was required for *in vitro* assembly of the RNAP holoenzyme and speculated that *R. capsulatus* may have an essential requirement for ω, in contrast to *E. coli* and other bacteria where it is not essential for growth [43, 69, 70]. Our results demonstrate that *R. capsulatus* does not require ω for laboratory growth, but we found that a loss of ω increased the doubling time of cultures (not shown). The RcGTA capsid protein and the lysis proteins are encoded separately in the chromosome of *R. capsulatus*, but their expression is co-regulated by the conserved response regulator CtrA [8, 31, 48, 50]. It therefore appears likely that ω is required for activation of the different RcGTA promoters; however we have not measured transcription directly.

Carbon depletion and ppGpp have been reported to influence CtrA levels in other Alphaproteobacteria (see reference [71] for a recent review). We suspect that nutrient depletion increases RcGTA production through the CtrA pathway in *R. capsulatus*, but have not been able to demonstrate this. We note that the complete loss of capsid production and cell lysis by the *rpoZ* mutation is similar to the effect of a loss of CtrA, the *rcc01865* gene product or the LexA-repressor [49, 50], and it appears possible that CtrA may interact with and require the ω protein for induction of RcGTA production, or alternatively that ω is required for CtrA production. Fig. 5 presents a summary of possible signalling pathways and key regulators of RcGTA described in this manuscript.

In conclusion, we found that production of the *R. capsulatus* GTA is stimulated by nutrient depletion, and that the sole RSH or RNAP ω subunit is required for maximal or detectable RcGTA production, respectively.

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Conflicts of interest
The authors declare that there are no conflicts of interest.

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