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ORIGINAL ARTICLE

The *Pseudomonas aeruginosa* product pyochelin interferes with *Trypanosoma cruzi* infection and multiplication *in vitro*

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Background: Bacteria are sources of numerous molecules used in treatment of infectious diseases. We investigated effects of molecules produced by 26 *Pseudomonas aeruginosa* strains against infection of mammalian cell cultures with *Trypanosoma cruzi*, the aetiological agent of Chagas disease.

Methods: Vero cells were infected with *T. cruzi* in the presence of wild-type *P. aeruginosa* supernatants or supernatants of mutants with defects in the production of various virulence, quorum sensing and iron acquisition factors. Quantification of *T. cruzi* infection (percentage of infected cells) and multiplication (number of amastigotes per infected cell) was performed and cell viability was determined.

Results: Wild-type *P. aeruginosa* products negatively affected *T. cruzi* infection and multiplication in a dose-dependent manner, without evident toxicity for mammalian cells. *PvdD*/*pchE* mutation (loss of the *P. aeruginosa* siderophores pyoverdine and pyochelin) had the greatest impact on anti–*T. cruzi* activity. Negative effects on *T. cruzi* infection by pure pyochelin, but not pyoverdine, or other *P. aeruginosa* exoproducts studied, were quantitatively similar to the effects of benznidazole, the current standard therapy against *T. cruzi*.

Conclusions: The *P. aeruginosa* product pyochelin showed promising activity against *T. cruzi* and might become a new lead molecule for therapy development.

Keywords: *Trypanosoma cruzi*, Chagas disease, *Pseudomonas aeruginosa*, drug discovery, therapy, pyochelin

Introduction

Chagas disease is a worldwide systemic and often chronic disease caused by the protozoan *Trypanosoma cruzi*. In Latin America, which is regarded as the endemic area for *T. cruzi* infection, at least 6 million people are estimated to be affected,[1,2] There is also an increasing incidence of Chagas disease in the USA and other non-endemic countries.[3,4] It is estimated that more than 300 000 individuals in the USA are infected, with up to 45 000 developing cardiomyopathies.[1–4] Due to severe side effects and limited efficacy, the current standards of therapy (benznidazole or 5-nitrofuran nifurtimox) are mainly recommended for treatment of the acute phase and early chronic infections.[5,6]

Antimicrobials are weapons microorganisms produce in order to stake out their claims in ecosystems with limited resources and a multitude of microbial inhabitants.[7] The bacterium *Pseudomonas aeruginosa* and the fungus *Aspergillus fumigatus* form such an ecosystem in the lungs of cystic fibrosis patients.[8,9] *P. aeruginosa* has been shown to produce molecules that interfere with *A. fumigatus* biofilm metabolism and growth.[10] Using supernatants from *P. aeruginosa* mutants comparison with wild-type, we recently described that the siderophore pyoverdine is the main antifungal molecule in a low-iron environment.[10] Here we used supernatants from the same *P. aeruginosa* wild-type and mutants and investigated the effects on *T. cruzi* infection and multiplication in search of new molecules that would ultimately help in treating Chagas disease.

Materials and methods

Materials

Benznidazole (BNZ), amphotericin B (AmB), pyoverdine (PYOV), *Pseudomonas* Quinolone Signal (PQS), Giemsa solution, Bouin's

fixative solution, 2,3-bis[2-methoxy-4-nitro-5-sulfophenyl]-2H-tetrazolium-5-carboxanilide inner salt (XTT) and menadione were purchased from Sigma-Aldrich (St. Louis, MO, USA). Pyochelin (PYOC) was purchased from Santa Cruz Biotechnology (Dallas, TX, USA). Foetal calf serum (FCS; catalogue number 16140071) and RPMI 1640 were obtained from Gibco (New York, NY, USA). The homoserine lactones 3-oxo-C12-HSL and C4-HSL were synthesized as described previously.[11,12]

Strains

PA14, a widely studied *P. aeruginosa* strain,[13] is the parental strain of all PA14 mutants studied here. The *T. cruzi* strain Y (TcY, TcII, ATCC 50832) was used.

PA14 mutants

All PA14 mutants investigated here, with the exception of the *lasI* and *pscC* mutants, were described previously. The *lasI* mutant is lacking 3-oxo-C12-HSL production,[14] while the *pscC* mutant is defective in all three secretion systems.[15]

P. aeruginosa culture filtrate production

PA14 wild-type and mutant planktonic culture filtrate (Pasup) were prepared as detailed previously.[10] In brief, RPMI 1640 medium (Lonza, Walkersville, MD, USA) was used and quantitated *P. aeruginosa* suspensions were grown for 24 h at 37°C. Culture supernatants were filtered (0.22 μm) after the growth period.

Isolation of trypanomastigotes

Culture-derived trypanomastigotes of the Y strain (TcII) were obtained from monolayers of Vero cells (cell line based on kidney epithelial cells from African green monkey, CCL-81; ATCC, Manassas, VA, USA) that had been infected at a ratio of 5:1 (trypanomastigotes:Vero cells). Vero cells were incubated at 37°C in RPMI 1640, enriched with 5% inactivated foetal bovine serum (FBS), supplemented with antibiotics (penicillin 500 μ/mL and streptomycin 0.5 mg/mL). Parasites were collected from the culture supernatants by centrifugation at 1000 *g* for 10 min and the sediment was suspended in RPMI with 5% FBS. Parasites were counted using a Neubauer chamber and the number was adjusted according to assay needs.

Infection with *T. cruzi*

Monolayers of Vero cells were prepared on Matrigel-coated eight-well chamber slides at a density of 2×10^5 cells/well and cultivated for 24 h at 37°C in a 5% carbon dioxide (CO_2) atmosphere. Infection was carried out at a target effector ratio of 1:5 (cell:parasite), with 24 h interaction time in the presence of drugs (AmB, BNZ), pure substances (PYOV, PYOC, PQS, oxo-C12-HSL or C4-HSL) or *P. aeruginosa* supernatants before replacing the medium with RPMI with 5% FBS without drugs, supernatants or parasites. Forty-eight hours after infection, cells were washed with phosphate-buffered saline, fixed with Boudin's solution and Giemsa stained. The number of infected cells (defined as at least one 1 amastigote per cell) as well as the number of amastigotes per infected cells (a measure of multiplication) was determined for each vision field by microscopy. About 25–40% of all cells in the control wells were found to be infected by TcY. For comparison with treated wells, control infections were regarded as 100%.

Cell metabolism

The viability of uninfected cells was determined by XTT metabolic assay.[16] Cells (10^5) were seeded into a 96-well plate and allowed to settle for 24 h at 37°C in 5% CO_2 and 80% humidity. After medium change, drugs (AmB, BNZ), pure substances (PYOV, PYOC, PQS, oxo-C12-HSL or C4-HSL) or *P. aeruginosa* supernatants were added and cells were incubated for 24 h at 37°C in 5% CO_2 and 80% humidity. After incubation cells were washed with phosphate-buffered saline, XTT/menadione (200 μg/mL and 40 μM, respectively) in RPMI with 5% FBS was added to each well and incubated at 37°C. Tests were evaluated using a plate reader (Opsys MR, DYNEX Technologies, Chantilly, VA, USA).

Statistical analysis

Results were analysed using Student's *t* test if two equal-size groups were compared or by Student's *t* test with Welch modification if the two groups showed unequal sample sizes.

Results

P. aeruginosa produces molecules that affect *T. cruzi* Y infection and multiplication

P. aeruginosa exports a wide range of molecules that can be investigated using its culture supernatants. We used cell-free supernatants produced by planktonically growing *P. aeruginosa* PA14 cells (PA14sup) as well as dilutions of these supernatants against infection of Vero cells with the *T. cruzi* strain TcY. AmB, a known inhibitor of *T. cruzi*, was used as a comparator.[17] Our results show that PA14sup in dilutions of up to 1:16 significantly interfered with *T. cruzi* infection (Figure 1A), as well as multiplication (Figure 1B), without affecting cell metabolism (Figure 1C). Vero cell metabolism was even modestly enhanced by PA14sup (Figure 1C).

Determination of *P. aeruginosa* products involved in *T. cruzi* Y inhibition

In order to define the *P. aeruginosa* product responsible for anti-trypanosoma activity, we used a set of 26 PA14 mutants with single or double gene defects in virulence factors, upstream quorum sensing factors that induce virulence factor production or molecules responsible for iron uptake.[10] We found that several PA14 mutants showed defects in anti-trypanosoma infection (Figure 2A) and multiplication (Figure 2B). For *T. cruzi* infection, mutants in *pvdD/pchE* (mutant 1), *rsmA* (mutant 8), *pchE* (mutant 16) and *hcnA* (mutant 24) showed the highest loss of activity (PA14 vs PA14 mutants, all $p \leq 0.001$). Next highest were mutants in *pqsA* (mutant 4), *lasR* (mutant 7) and *phzC1/C2* (mutant 15) (PA14 vs PA14 mutants, all $p \leq 0.01$) and *HSI 1/2* (mutant 12), *chiC* (mutant 22) and *lecA* (mutant 23) (PA14 vs PA14 mutants, all $p \leq 0.05$). For *T. cruzi* multiplication, mutants *pvdD/pchE* (mutant 1), *pqsA* (mutant 4), *rsmA* (mutant 8), *pchE* (mutant 16) and *hcnA* (mutant 24) showed the largest loss

Table 1. PA14 mutants with significant loss of anti–*T. cruzi* activity

No.	Mutant	Mutation result	Reference
1	*pvdD-/pchE-*	Pyoverdine–pyochelin double siderophore mutant	10
4	*pqsA-*	Gene for anthranilate-CoA ligase lost; loss of extracellular quinolones, HAQ biosynthesis, including HHQ, PQS and DHQ, thus absent or decreased activity of MvfR	34
7	*lasR-*	Lacks several QS-regulated factors, including proteases, 3-oxo-C12-HSL; delayed activation of RhlR QS pathway, less pyoverdine	17
8	*rsmA-*	Global post-transcriptional regulator mutant	35
12	*HSI 1/2*	Double mutant defective in two of three type VI secretion systems	36
15	*phzC1-/phzC2-*	Double phenazine mutant (completely abrogated), no pyocyanin	–[a]
16	*pchE-*	Loss of pyochelin (siderophore) mutant	18
21	*pqsA-pqsH- polar*	ED2, pqsA::TnPhoA, pqsH::Gm, the mutation in pqsA is polar, kan and gm resistant	–[a]
22	*chiC-*	Loss of chitinase	18
23	*lecA-*	Loss of galactose-specific lectin A	18
24	*hcnA-*	No hydrogen cyanide production	18
26	*lasI-*	No 3-oxo-C12-HSL production	14

[a]Unpublished lab strain (available from E. Deziel).

of activity (PA14 vs PA14 mutants, all $p \leq 0.001$). Next highest were *phzC1/C2* (mutant 15 vs PA14, $p \leq 0.01$), *lasR* (mutant 7), *pqsA/pqsH* polar (mutant 21), *lecA* (mutant 23) and *lasI* (mutant 26) (PA14 vs PA14 mutants, all $p \leq 0.05$). All mutants showing significant losses in anti-trypanosoma activity are detailed in Table 1. The combined loss of both pyoverdine and pyochelin (*pvdD/pchE* mutant 1) seemed to increase *T. cruzi* multiplication over measurements obtained in control wells (Figure 2B). AmB was toxic for Vero cells, but none of the supernatants showed such toxicity (Figure 2C). In fact, most supernatants, with the exception of supernatants for *rsmYZ* (mutant 18), increased Vero cell metabolism (Figure 2C).

Pyochelin affects *T. cruzi* Y infection and multiplication

As detailed in Table 1, a variety of *P. aeruginosa*–produced molecules could be responsible for anti–*T. cruzi* effects. To examine the effects of *P. aeruginosa*–produced molecules on *T. cruzi* directly and compare them to the currently licensed anti-trypanosome drug BNZ,[5] we investigated the anti–*T. cruzi* effects of pure pyochelin, pyoverdine, PQS, C4-HSL and 3-oxo-C12-HSL. Our results show that at 10 μM, only pyochelin reduced *T. cruzi* infection to levels that were achievable by the *in vitro* equivalent to the therapeutic serum range of benznidazole (11.5–23 μM) (Figure 3A). Lesser concentrations of pyochelin did not affect *T. cruzi* infection (data not shown). Pyochelin also reduced *T. cruzi* multiplication, but not to levels achievable by benznidazole (Figure 3B). When *T. cruzi* was incubated with pyochelin prior to infection for 2 h, neither infection nor multiplication was affected (data not shown), suggesting that pyochelin is not directly toxic for *T. cruzi*. Pyoverdine did not affect *T. cruzi* infection (Figure 3A), but increased *T. cruzi* multiplication above levels observed in control wells (Figure 3B). The quorum-sensing signals PQS, C4-HSL and 3-oxo-C12-HSL did not affect *T. cruzi* infection or its multiplication (Figures 3A and B). Vero cell metabolism was slightly affected by pyoverdine, and to a greater degree by PQS, but not by pyochelin (Figure 3C). C4-HSL and 3-oxo-C12-HSL stimulated Vero cell metabolism (Figure 3C).

Discussion

For a long time, chemotherapy for *T. cruzi* infection, and the resulting Chagas disease, has been limited to benznidazole and nifurtimox. Both are predominantly used in the treatment of acute and early chronic-phase Chagas disease, owing to severe side effects with high doses and long-term application.[5,6] New potential treatments under investigation, either alone or in combination, include inhibitors of ergosterol biosynthesis, calcium homeostasis, trypanothione metabolism, cysteine proteases, pyrophosphate metabolism, protein and purine synthesis, as well as lysophospholipid analogues (LPAs) and natural substances.[18] Certain azoles, such as posaconazole and itraconazole, were promising candidates for anti-trypanosoma therapy in clinical studies, but appear not to be curative,[6] whereas amphotericin B prolonged life in mice and rapidly cleared the blood of all parasites but did not clear tissue infection.[17]

Most antibiotics that are used in humans are produced by microbes.[19] *P. aeruginosa* is less well-known as a source for antibiotics but produces a multitude of antimicrobials in order to survive in microenvironments with competition for limited resources.[7] An example of such a competitive ecosystem is the lungs of cystic fibrosis patients, where the bacterium *P. aeruginosa* and the fungus *A. fumigatus* fight for resources.[8,9,20] *P. aeruginosa* has been shown to produce molecules that interfere with *A. fumigatus* biofilm metabolism and growth, of which the siderophore pyoverdine is a main antifungal molecule.[10]

In the present study, we found that exported products from *P. aeruginosa* also have a negative impact on *T. cruzi* infections, without affecting mammalian cells. In contrast to PA14

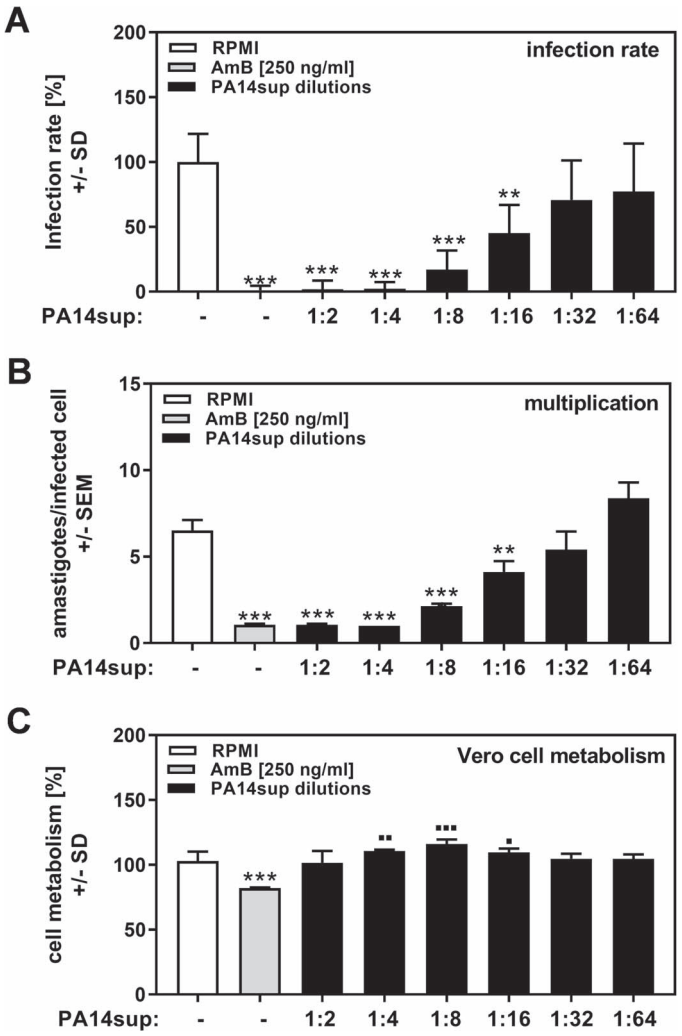

Figure 1. *P. aeruginosa* molecules affect *T. cruzi* Y strain infection and multiplication. Vero cells (2×10^5/well) were infected with TcY at a ratio of 5 trypanosoma per cell for 24 h at 37°C in 5% CO_2 and 80% humidity. Infections took place in the presence of pure RPMI, AmB (250 ng/mL) or 1:2 dilutions of planktonic PA14 wild-type cell-free supernatants (1:2 to 1:64). Twenty-four hours after infection, cells were washed and fresh medium without PA14 supernatants or TcY was added. (**A**) Infection rates and (**B**) multiplication were determined 48 h after infection. (**C**) Cell viability was determined by XTT assay. Statistics: Welch *t* test; comparison, RPMI vs all other bars. Asterisks represent significant decreases, squares represent significant increases. One, two or three asterisks or squares: $p \leq 0.05$, $p \leq 0.01$ or $p \leq 0.001$, respectively.

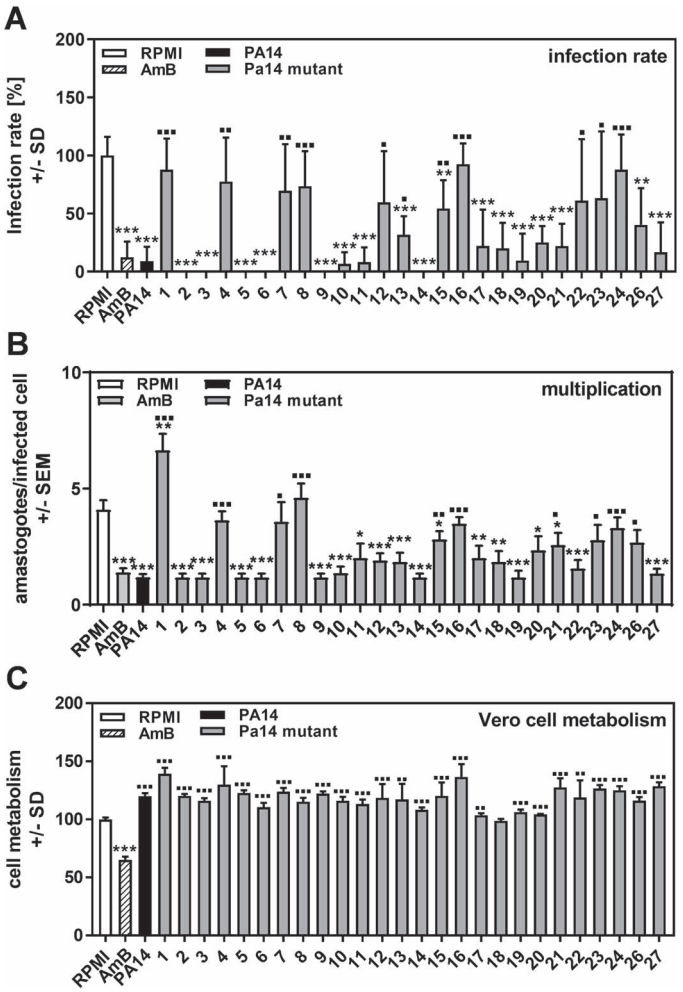

Figure 2. Determination of *P. aeruginosa* products involved in *T. cruzi* Y strain inhibition. Vero cells (2×10^5/well) were infected with TcY at a ratio of 5 trypanosoma per cell for 24 h at 37°C in 5% CO_2 and 80% humidity. Infections took place in the presence of pure RPMI, AmB (250 ng/mL) or 1:2 dilutions of planktonic PA14 wild-type or mutant cell-free supernatants. Twenty-four hours after infection, cells were washed and fresh medium without supernatants or TcY was added. (**A**) Infection rates and (**B**) multiplication were determined 48 h after infection. (**C**) Cell viability was determined by XTT assay. Statistics: Welch *t* test; comparisons (A and B): asterisks: RPMI vs all other bars; squares: PA14 wild-type supernatant (black bar) vs all other bars; (C): asterisks represent significant decreases, squares represent significant increases. One, two or three asterisks or squares: $p \leq 0.05$, $p \leq 0.01$ or $p \leq 0.001$, respectively. Mutants: 1: *pvdD-pchE-*; 2: *pqsE-*; 3: *mvfR-*; 4: *pqsA-*; 5: *pqsH-*; 6: *lasR-rhlR-*; 7: *lasR-*; 8: *rsmA-*; 9: *pqsA-pqsH-* (not polar); 10: *pvdD-*; 11: *rhlR-*; 12: *HSI-1/2-*; 13: *pvcA-*; 14: *rhlA-*; 15: *phzC1-phzC2-*; 16: *pchE-*; 17: *exoU-*; 18: *rsmY-rsmZ-*; 19: *HSI-2/3-*; 20: *HSI-1/3-*; 21: *pqsA-pqsH-* (polar); 22: *chiC-*; 23: *lecA-*; 24: *hcnA-*; 26: *lasI-*, 27: *pscC-*.

wild-type, several PA14 mutants were unable to inhibit *T. cruzi* infection and multiplication, suggesting that they lacked production of the active compounds. The loss of *pvdD/pchE*, *rsmA*, *pchE* and *hcnA* showed the greatest impact on anti–*T. cruzi* activity, followed by the loss of *pqsA*, *lasR, phzC1/C2*, *pqsA/pqsH* (polar) and *lasI*.

Iron is a crucial nutrient for *P. aeruginosa*, as well as for many other organisms, including *A. fumigatus* and *T. cruzi*.[10,21,22] Pyoverdine and pyochelin are the major siderophores of *P. aeruginosa*. It has been shown that pyoverdine-bound iron is not available for *A. fumigatus*,[10] while it is not yet known if pyochelin acts in the same way or if *T. cruzi* can use or is affected by pyoverdine- or pyochelin-bound iron. The loss of both the siderophores pyochelin and pyoverdine, or pyochelin alone, significantly interfered with *T. cruzi* infection and multiplication, while the loss of pyoverdine alone had no effect. Anti–*T. cruzi*

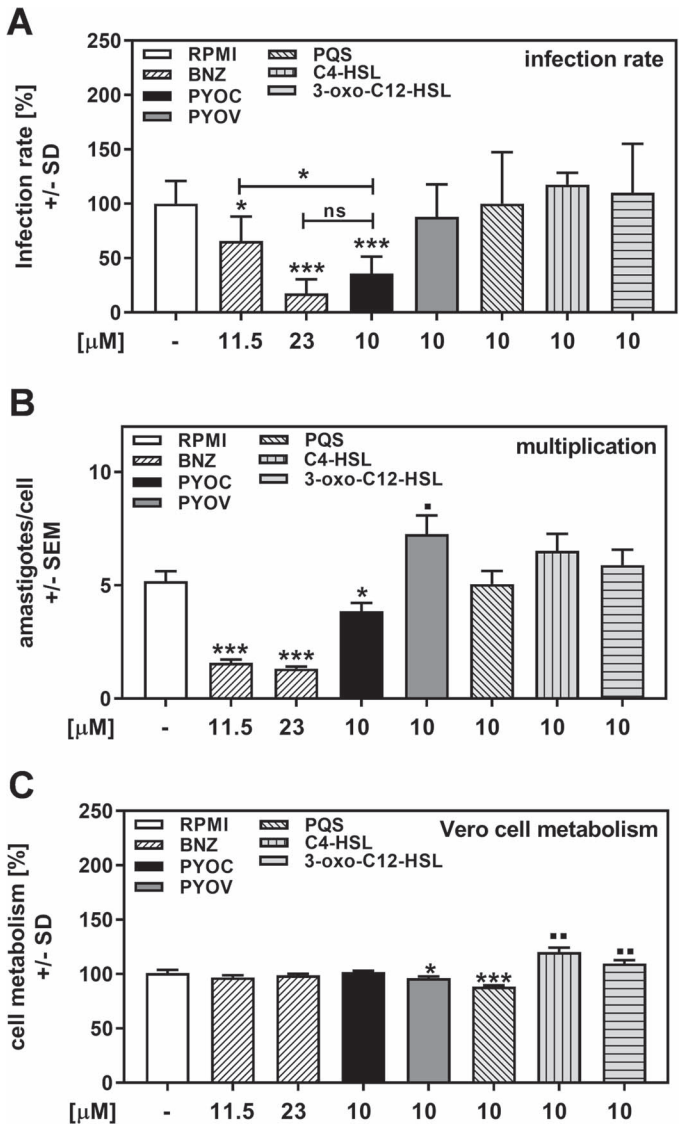

Figure 3. Pyochelin affects *T. cruzi* Y strain infection and multiplication. Vero cells (2×10^5/well) were infected with TcY at a ratio of 5 trypanosoma per cell for 24 h at 37°C in 5% CO_2 and 80% humidity. Infections took place in the presence of pure RPMI, BNZ (11.5 or 23 μM), pyochelin (PYOC, 10 μM), pyoverdine (PYOV, 10 μM), PQS (10 μM), C4-HSL (10 μM) or 3-oxo-C12-HSL (10 μM). Twenty-four hours after infection, cells were washed and fresh medium without drugs or TcY was added. (**A**) Infection rates and (**B**) multiplication were determined 48 h after infection. (**C**) Cell viability was determined by XTT assay. Statistics: Welch *t* test. Comparisons: RPMI vs all other bars. Asterisks represent significant decreases, squares represent significant increases. One, two or three asterisks or squares: $p \leq 0.05$, $p \leq 0.01$ or $p \leq 0.001$, respectively. ns: not significant.

activity could also be replicated by treatment with pyochelin, while pyoverdine addition showed no inhibition. Consequently, although both pyochelin and pyoverdine are *P. aeruginosa* siderophores and involved in iron acquisition, only pyochelin was active against *T. cruzi* infection. Pyoverdine and pyochelin bind ferric iron, but pyoverdine binds with greater affinity.[23] Earlier reports showed that iron is a crucial factor for intracellular growth of *T. cruzi* in macrophages[21] and for infection in mice.[22] Therefore one possible mechanism of pyochelin action is limiting iron availability for *T. cruzi* by binding available iron in the medium. However, since pyoverdine, the better iron chelator, has no effect, this hypothesis is unlikely. Pyochelin also binds other metals, like zinc and copper.[23,24] Our current experiments cannot rule out that binding of these metals affects *T. cruzi* infection.

Pyochelin-bound iron causes oxidative damage and inflammation, especially in the presence of pyocyanin, another compound exported by *P. aeruginosa*.[25,26] For example, pyochelin-bound iron enhances the production of intracellular reactive oxygen species (ROS) in *Enterococcus faecalis* over time, causing a significant increase in lipid peroxidation and cell death by disrupting the integrity of the bacterial membrane.[27] It is possible that pyochelin might induce oxidative stress to inhibit *T. cruzi*. The *phzC1/C2* mutant cannot produce phenazines, including the redox-active pyocyanin.[28] The *phzC1/C2* mutant lost considerable anti–*T. cruzi* activity, supporting a potential mechanism of pyochelin and pyocyanin inducing oxidative stress in *T. cruzi*. Whereas excessive oxidative stress is toxic for *T. cruzi*, it can be mitigated by the *T. cruzi* aspartic proteinase TcAP1.[29] Moderate oxidative stress is beneficial for *T. cruzi* multiplication,[30] however, pyochelin demonstrated inhibition of multiplication as well. Further studies will be required to support an oxidative stress mode of action.

Other mutations also led to a loss in the anti–*T. cruzi* activity of PA14 supernatants, suggesting that additional *P. aeruginosa* compounds may also be effective inhibitors. PqsA is an enzyme involved in the synthesis of 4-hydroxy-2-alkylquinolines (HAQs), including PQS. Mutation of *pqsA* prevents HAQ production, leading to decreased activity of MvfR, a key transcriptional regulator in the quorum sensing pathway that is required for full *P. aeruginosa* virulence.[31] While both the loss of *pqsA* and *pqsA/pqsH* (polar) also resulted in a loss of anti–*T. cruzi* activity, the *mvfR*- mutant did not result in reduced anti–*T. cruzi* activity of *P. aeruginosa* supernatants, nor did the addition of pure PQS affect *T. cruzi*. It would be worthwhile testing other HAQs produced by the *pqsA* pathway for anti–*T. cruzi* activity.

The other key autoinducers of the quorum sensing pathway are 3-oxo-C12-HSL and C4-HSL, where detection by LasR and RhlR, respectively, leads to downstream production of virulence factors like toxins and proteases in addition to regulating swarming and biofilm activity.[32] LasI is a synthase that produces 3-oxo-C12-HSL. While the *lasI* mutant showed a reduction of anti–*T. cruzi* activity, the addition of 3-oxo-C12-HSL or C4-HSL had no effect. The *lasR* mutant suffered a greater loss in anti–*T. cruzi* activity, suggesting that one of the downstream virulence factors produced by the pathway may have additional anti–*T. cruzi* effects.

Mutations in *rsmA*, *hcnA*, *chiC*, *lec* and *HSI 1/2* also had an impact on *P. aeruginosa* activity against *T. cruzi* and may reflect other factors that could participate in anti–*T. cruzi* action.

Almost all the *P. aeruginosa* supernatants, whether from wild-type or a mutant, increased the metabolism of Vero cells. The reason for this might be traces of *Pseudomonas*-produced lipopolysaccharide (LPS) in the bacterial supernatants.[33] LPS is a well-known stimulator of mammalian cell proliferation, but it does not stimulate *T. cruzi* multiplication.[34]

In our study, infection and treatment took place at the same time. Although preliminary results indicate that 2 h of pre-incubating *T. cruzi* Y strain with pyochelin does not have significant effects on infection, it might be possible that prolonged incubation affects the parasite. Another possibility would be that pyochelin inhibits *T. cruzi* growth at the stage of the epimastigote proliferative form.

Although the results presented here are preliminary, the *P. aeruginosa* product pyochelin has significant anti–*T. cruzi* Y strain activity, partially in the same range as therapeutic concentrations of benznidazole. Whether pyochelin could become a future lead substance for therapeutic drug development against Chagas disease will also depend on its performance in *in vivo* studies that determine its selectivity index.[35] Our results also suggest that there are other *P. aeruginosa* molecules with anti–*T. cruzi* activity that are yet to be defined.

Authors' contributions:

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