AMERICAN SOCIETY FOR MICROBIOLOGY | Antimicrobial Agents and Chemotherapy®

MECHANISMS OF RESISTANCE

Role of Whole-Genome Sequencing in Characterizing the Mechanism of Action of *para*-Aminosalicylic Acid and Its Resistance

Giovanni Satta,[a,b] Adam A. Witney,[c] Neelu Begum,[a] Julio Ortiz Canseco,[a] Andrew N. Boa,[d] Timothy D. McHugh[a]

[a]Centre for Clinical Microbiology, University College London, London, United Kingdom
[b]Department of Infectious Disease, Imperial College London, London, United Kingdom
[c]Institute for Infection and Immunity, St. George's University of London, London, United Kingdom
[d]Department of Chemistry, University of Hull, Hull, United Kingdom

ABSTRACT *para*-Aminosalicylic acid (PAS) remains one of the drugs of last resort for the treatment of tuberculosis, but its mechanism of action is still not completely understood. The main aim of this project was to identify new potential mechanisms of action and resistance to PAS by performing whole-genome sequencing on PAS-resistant laboratory mutants. A new variant in the *folC* gene was identified, as well as some other mutations that require further study.

KEYWORDS *para*-aminosalicylic acid, whole-genome sequencing, tuberculosis

para-Aminosalicylic acid (PAS), also known as 4-aminosalicylic acid, was one of the first chemotherapeutic agents to be used against tuberculosis (TB), and it is currently an orphan drug available to treat only extensively drug-resistant disease (1). Despite being used for decades, its mechanism of action is not completely understood. It has been proposed that, being an analogue of *para*-aminobenzoic acid (PABA), PAS competes with PABA for dihydropteroate synthase, interfering in the process of folate synthesis (2). A study using transposon mutagenesis identified mutations in the *thyA* gene that were also present in clinical isolates resistant to PAS (3). The gene *thyA* encodes a thymidylate synthase enzyme (essential for DNA replication and repair), and its deletion has been demonstrated to confer resistance to PAS (4). Other studies have identified various missense mutations in *folC* (encoding a dihydrofolate synthase) and *ribD* (alternative dihydrofolate reductase) that conferred resistance to PAS in laboratory and clinical isolates of *Mycobacterium tuberculosis* (5–7). Nevertheless, mutations in *folC* were detected in only 34.8% of resistant clinical isolates, whereas mutations of *thyA* and *ribD* were detected in 26.0% and 5.8%, respectively (6). Hence, other mechanisms of resistance to the drug might exist. Efflux pumps have been described conferring cross-resistance to PAS and other chemotherapeutic agents, including streptomycin (8). The main aim of this work was to investigate potential new mechanisms of action and resistance to PAS by performing whole-genome sequencing (WGS) on PAS-resistant laboratory mutants.

PAS-resistant laboratory mutants of reference strain H37Rv were spontaneously selected by growth on 7H10 medium with the addition of PAS. *M. tuberculosis* isolates were cultured in 20-ml aliquots of sterile 7H9 broth and incubated for 14 days, achieving log phase and a colony count of 0.5 to 1 McFarland (150 to 300 $\times$ 10^6/ml). After the incubation period, the bacteria were concentrated by centrifugation at 10,000 $\times$ *g* for 10 minutes, and the entire sediment was inoculated onto preprepared 7H10 plates containing different concentrations, i.e., 2 and 4 μg/ml, of PAS (the critical concentration for PAS is 2 μg/ml) (9). After incubation at 37°C for at least 14 days,

Citation Satta G, Witney AA, Begum N, Ortiz Canseco J, Boa AN, McHugh TD. 2020. Role of whole-genome sequencing in characterizing the mechanism of action of *para*-aminosalicylic acid and its resistance. Antimicrob Agents Chemother 64:e00675-20. https://doi.org/10.1128/AAC.00675-20.

Address correspondence to Giovanni Satta, giovanni.satta@nhs.net.

Received 18 April 2020
Returned for modification 4 June 2020
Accepted 13 June 2020
Accepted manuscript posted online 22 June 2020
Published 20 August 2020

TABLE 1 Genes involved in the PAS-resistant mutants, with the respective function and SNP position in the genome

Gene[a]	Function	SNP[b] position for:			
		PAS2		PAS4	
		1st	2nd	1st	2nd
Rv1392 (*metK*)	*S*-adenosylmethionine synthetase			1566981	
Rv2447c (*folC*)	Folylpolyglutamate synthase			2747195 (D135G)	2747141 (E153A)
Rv3218	Hypothetical protein	3594639[c] (V58I)	3594639[c] (V58I)	3594639 (V58I)	
Rv3759c (*proX*)-*Rv3760* intergenic	Possible osmoprotectant binding lipoprotein; conserved membrane protein				4205442

[a]At low coverage, a total of 27 SNPs were found in the *rrs* and *rrl* genes (16s and 23s RNA genes) of all four mutants. Data not shown because of low coverage.
[b]Only high-quality nonsynonymous and intergenic SNPs were considered. Hypothetical amino acid changes caused by SNP are shown in parentheses.
[c]Mixed base calls and therefore lower-quality evidence in these mutants for these sites.

spontaneous mutants grew on the plates and were then selected for sequencing. All selected mutants and the parent reference strain H37Rv were subcultured on Lowenstein-Jensen slopes, DNA was extracted using the CTAB (cetyltrimethylammonium bromide) method, and WGS analysis was performed as previously described (10).

It was possible to grow only one PAS mutant for each critical concentration (2 and 4 μg/ml) from a culture containing 150 to 300 $\times$ 10^6 bacteria/ml. Because the experiment was repeated on two separate occasions, we were able to select a total of four mutants. These resistant mutants were designated PAS2 (1st and 2nd positions) and PAS4 (1st and 2nd positions).

Compared to the sequenced reference strain H37Rv, a total of seven nonsynonymous single-nucleotide polymorphisms (SNPs) affecting four different genes were identified in the four PAS-resistant mutants. Both PAS4 mutants showed variants in the *folC* gene, a known mutation in position 2747141 and a new mutation in position 2747195 (Table 1). There was evidence of a V58I variant in the *Rv3218* gene in both of the PAS2 and one of the PAS4 mutants, but reads matching both the reference and variant base were found at this site, and this call is uncertain. At low coverage, a total of 27 SNPs were also found in the *rrs* and *rrl* genes (16s and 23s RNA genes) of all four mutants. However, these SNPs remain unconfirmed because of the low coverage.

WGS has been used to determine the mechanism of action of antituberculous agents. In the case of bedaquiline (BDQ), researchers selected and sequenced BDQ-resistant *Mycobacterium smegmatis* strains and identified mutations in the proton pump of ATP synthase associated with resistance (11). In the case of PAS-resistant mutants, WGS analysis indicated that it is necessary to revisit the folate metabolic pathway to fully understand our data. The folate biosynthetic pathway starts when the aromatic precursor chorismate is converted to PABA and coupled with pteridine to generate dihydropteroate. The protein encoded by *folC*, dihydrofolate synthetase, adds glutamate to the dihydropteroate forming dihydrofolate (12). Mutations in the *folC* gene have been found to be associated with PAS resistance but in $<$35% of the cases, but the same researchers did not find any mutation in the *folP1* gene (4). Here, both PAS4-resistant mutants gained an SNP affecting the *folC* gene (Table 1). Mutation E153A has been reported to confer resistance to PAS (12), and it is already included in some online databases for WGS analysis. The new mutation, D135G, was not previously associated with PAS resistance. However, it has been shown to be important for the linkage of α-helices in the *folC* protein structure (5), and it may represent an additional mechanism of resistance to PAS. It is interesting to note that such variants in the *folC* gene did not develop in the PAS2 mutants at lower concentrations, raising the hypothesis of an association with high-level resistance as noted in other antituberculous drugs (13). This may have some clinical relevance in that higher levels of resistance may be managed and potentially prevented by optimizing the dosage as previously described by other authors (14). It is also worth highlighting the fascinating (albeit unconfirmed) finding of *rrs* and *rrl* gene mutations. These genes are linked to the ribosomes, including the conversion from tRNAMet to tRNAfMet (Fig. 1), and hence the synthesis of proteins in general. This would hypothetically resemble the mechanism of

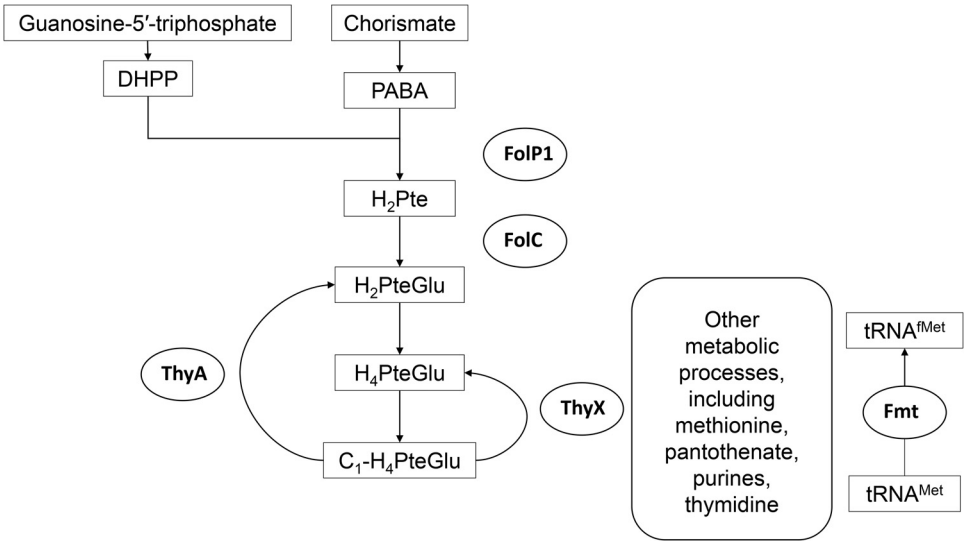

FIG 1 Folate metabolism in *M. tuberculosis*. DHPP, 7,8-dihydropterin pyrophosphate; PABA, *para*-aminobenzoic acid; H_2Pte, dihydropteroate; H_2PteGlu, dihydrofolate; H_4PteGlu, tetrahydrofolate; C1-H_4PteGlu, various single-carbon-modified species of H_4PteGlu; ThyX, thymidylate synthase; tRNA^{Met}, methionyl-tRNA; tRNA^{fMet}, *N*-formyl-methionyl-tRNA; Fmt, formyl-methionine transferase.

resistance of streptomycin, even if streptomycin resistance is more frequently associated with mutations in the *rpsL* gene rather than *rrs/rrl* (15). Because PAS and streptomycin have been companion drugs for decades, and at a time when next-generation sequencing technologies were not available, this mechanism of action/resistance may have been missed and is certainly worth further research. Our analysis is based on only four resistant mutants because it was difficult to select PAS-resistant mutants in the laboratory. This may be because of the essentiality of genes involved and the negative impact that a deletion and/or an altered gene function would have on cell survival (16). In addition, the presence of mutations in the genome needs additional confirmation that such mutations encode significant metabolic changes.

After nearly 70 years of clinical use of PAS, WGS analysis may help in elucidating its mechanism of action, but further studies are still needed.

Data availability. The sequence data generated have been deposited in the European Nucleotide Archive database hosted by The European Bioinformatics Institute under BioProject accession no. PRJEB36463 (SRA accession no. ERP119659).

ACKNOWLEDGMENTS

The experiments described in the manuscript were performed as part of G.S.'s PhD research at University College London using internal departmental funds.

We have no conflicts of interest to declare.

REFERENCES

1. Donald PR, Diacon AH. 2015. *para*-Aminosalicylic acid: the return of an old friend. Lancet Infect Dis 15:1091–1099. https://doi.org/10.1016/S1473-3099(15)00263-7.
2. Minato Y, Thiede JM, Kordus SL, McKlveen EJ, Turman BJ, Baughn AD. 2015. *Mycobacterium tuberculosis* folate metabolism and the mechanistic basis for *para*-aminosalicylic acid susceptibility and resistance. Antimicrob Agents Chemother 59:5097–5106. https://doi.org/10.1128/AAC.00647-15.
3. Mathys V, Wintjens R, Lefevre P, Bertout J, Singhal A, Kiass M, Kurepina N, Wang XM, Mathema B, Baulard A, Kreiswirth BN, Bifani P. 2009. Molecular genetics of *para*-aminosalicylic acid resistance in clinical isolates and spontaneous mutants of *Mycobacterium tuberculosis*. Antimicrob Agents Chemother 53:2100–2109. https://doi.org/10.1128/AAC.01197-08.
4. Fivian-Hughes AS, Houghton J, Davis EO. 2012. *Mycobacterium tuberculosis* thymidylate synthase gene *thyX* is essential and potentially bifunctional, while *thyA* deletion confers resistance to *p*-aminosalicylic acid. Microbiology 158:308–318. https://doi.org/10.1099/mic.0.053983-0.
5. Zhao F, Wang XD, Erber LN, Luo M, Guo AZ, Yang SS, Gu J, Turman BJ, Gao YR, Li DF, Cui ZQ, Zhang ZP, Bi LJ, Baughn AD, Zhang XE, Deng JY. 2014. Binding pocket alterations in dihydrofolate synthase confer resistance to *para*-aminosalicylic acid in clinical isolates of *Mycobacterium tuberculosis*. Antimicrob Agents Chemother 58:1479–1487. https://doi.org/10.1128/AAC.01775-13.
6. Zhang X, Liu L, Zhang Y, Dai G, Huang H, Jin Q. 2015. Genetic determinants involved in *p*-aminosalicylic acid resistance in clinical isolates from tuberculosis patients in northern China from 2006 to 2012. Antimicrob Agents Chemother 59:1320–1324. https://doi.org/10.1128/AAC.03695-14.

7. Wei W, Yan H, Zhao J, Li H, Li Z, Guo H, Wang X, Zhou Y, Zhang X, Zeng J, Chen T, Zhou L. 2019. Multi-omics comparisons of p-aminosalicylic acid (PAS) resistance in folC mutated and un-mutated Mycobacterium tuberculosis strains. Emerg Microbes Infect 8:248–261. https://doi.org/10.1080/22221751.2019.1568179.
8. Ramón-García S, Mick V, Dainese E, Martín C, Thompson CJ, De Rossi E, Manganelli R, Aínsa JA. 2012. Functional and genetic characterization of the tap efflux pump in *Mycobacterium bovis* BCG. Antimicrob Agents Chemother 56:2074–2083. https://doi.org/10.1128/AAC.05946-11.
9. Heifets L. 2013. Role of phenotypic methods for drug susceptibility testing of M. tuberculosis isolates in the era of MDR and XDR epidemics. In McHugh TD (ed), Tuberculosis: laboratory diagnosis and treatment strategies. CABI, London, United Kingdom.
10. Satta G, Witney AA, Shorten RJ, Karlikowska M, Lipman M, McHugh TD. 2016. Genetic variation in Mycobacterium tuberculosis isolates from a London outbreak associated with isoniazid resistance. BMC Med 14:117. https://doi.org/10.1186/s12916-016-0659-6.
11. Andries K, Verhasselt P, Guillemont J, Göhlmann HW, Neefs JM, Winkler H, Van Gestel J, Timmerman P, Zhu M, Lee E, Williams P, de Chaffoy D, Huitric E, Hoffner S, Cambau E, Truffot-Pernot C, Lounis N, Jarlier V. 2005. A diarylquinoline drug active on the ATP synthase of Mycobacterium tuberculosis. Science 307:223–227. https://doi.org/10.1126/science.1106753.
12. Green JM, Matthews RG. 2007. Folate biosynthesis, reduction, and polyglutamylation and the interconversion of folate derivatives. EcoSal Plus 2. https://doi.org/10.1128/ecosalplus.3.6.3.6.
13. Lempens P, Meehan CJ, Vandelannoote K, Fissette K, de Rijk P, Van Deun A, Rigouts L, de Jong BC. 2018. Isoniazid resistance levels of Mycobacterium tuberculosis can largely be predicted by high-confidence resistance-conferring mutations. Sci Rep 8:3246. https://doi.org/10.1038/s41598-018-21378-x.
14. Sy SK, de Kock L, Diacon AH, Werely CJ, Xia H, Rosenkranz B, van der Merwe L, Donald PR. 2015. *N*-acetyltransferase genotypes and the pharmacokinetics and tolerability of *para*-aminosalicylic acid in patients with drug-resistant pulmonary tuberculosis. Antimicrob Agents Chemother 59:4129–4138. https://doi.org/10.1128/AAC.04049-14.
15. Springer B, Kidan YG, Prammananan T, Ellrott K, Bottger EC, Sander P. 2001. Mechanisms of streptomycin resistance: selection of mutations in the 16S rRNA gene conferring resistance. Antimicrob Agents Chemother 45:2877–2884. https://doi.org/10.1128/AAC.45.10.2877-2884.2001.
16. DeJesus MA, Gerrick ER, Xu W, Park SW, Long JE, Boutte CC, Rubin EJ, Schnappinger D, Ehrt S, Fortune SM, Sassetti CM, Ioerger TR. 2017. Comprehensive essentiality analysis of the *Mycobacterium tuberculosis* genome via saturating transposon mutagenesis. mBio 8:e02133-16. https://doi.org/10.1128/mBio.02133-16.