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Salicylic acid is not a bacterial siderophore: a theoretical study

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Abstract

Using a newly available program for calculating the concentrations and speciation of various ions (Pettit, LD & Powell KJ, 'SolEq' Academic Software, 1999), we have calculated that at pH 7 the amount of free Fe(III) present in an aqueous solution is 1.4×10^{-9} M and not 10^{-18} M as is usually quoted. In the presence of salicylic acid, included in the calculations at 10^{-4} M, the solubility of Fe(III) is increased to only 9.8×10^{-9} M suggesting that salicylate is unable to act as a siderophore although it is produced as an extracellular product by several bacterial genera when grown iron deficiently. In the presence of 40 mM phosphate, the soluble Fe(III) concentration is decreased by 10^4 at pH 7 and again this is hardly affected by the presence of salicylate. Thus, for microorganisms grown either *in vitro* or *in vivo*, salicylate is unlikely to function as a iron solubilizing agent. The same conclusions may also apply to 2,3-dihydroxybenzoic acid.

Introduction

Salicylic acid (2-hydroxybenzoic acid) occurs in extracellular culture filtrates of several bacteria when they have been grown with a deficiency of iron: organisms include *Pseudomonas* and *Burkholdia* spp. (Meyer, 1992; Meyer *et al.* 1992; Visca *et al.* 1993), *Azospirillum lipoferum* (Saxena *et al.* 1986) and *Mycobacterium* spp. (Ratledge & Winder 1962). The salicylate moiety also occurs in yersiniabactin, the siderophore of *Yersinia* spp. (Drechsel *et al.* 1995; Chambers *et al.* 1996), and in other siderophores such as vulnibactin from *Vibrio vulnificus* and parabactin from Paracoccus denitrificans (see Drechsel & Winkelmann 1997). The suggestion has been made that salicylate can act as a siderophore (FeIII-chelating substance) in its own right (Sokol *et al.* 1999; Visca *et al.* 1993; Meyer 1992) serving to transport iron into the bacteria. Ratledge *et al.* (1974), however, showed that salicylate was ineffective in holding ferric ions in solution at pH 7 in the presence of phosphate ions which are, of course, ubiquitous in bacterial culture media as well as being present in animal tissues and fluids at between 1 to 6 mM (Long 1961) where many of these organisms, as pathogenic bacteria, will reside. This observation though has not prevented these other, more recent, claims being made.

It is relevant to point out that the related acid, 2,3-dihydroxybenzoic acid, also occurs as a free entity in cultures of *Escherichia coli* and related bacteria when also grown iron-deficiently (O'Brien & Gibson 1970) and claims again have been made (Lopez Goni *et al.* 1992, 1995) that this too could act as a siderophore.

The purpose of this paper is to show that, on theoretical grounds, there is no evidence that salicylic acid is capable of holding Fe(III) effectively in solution at pH values above 6 and that, only below pH 5, which is unrealistic for most bacterial cultures, would salicylate be able to hold appreciable amounts of iron in solution. In this study we have been concerned only with Fe(III). There are clearly no problems with the solubility of Fe(II) though anoxic conditions are obviously needed for its uptake into bacteria.

Methods

Program and data used

The database program *SolEq* of Pettit & Powell (1999) was used in conjunction with data from Martell & Smith (1977, 1987) and Smith & Martell (1976, 1989). The program *Species* (included with *SolEq*) was used to calculate speciation variation with pH.

Salicylic acid was originally included in the program at a nominal concentration of 10^{-4} M (~ 14 mg l^{-1}) which is about the concentration that it has been recorded with mycobacteria (Ratledge & Winder 1962) though concentrations of up to 5×10^{-4} M have been recorded under certain other conditions (Adilakshmi *et al.* 2000).

Iron was included in the programme at 10^{-6} M as this is the usual concentration of iron that must be used to achieve iron deficient growth conditions with mycobacteria (Adilakshmi *et al.* 2000) though lower concentrations, 10^{-7} M, may be necessary with other bacteria to achieve iron deficient conditions (Visca *et al.* 1993). Higher concentrations of iron, 4×10^{-5} M, would repress salicylate biosynthesis (Ratledge & Winder 1962).

pH values have extended across the complete range of 1 to 14 though it is unusual for bacteria to grow outside the range of 5.5 to 9.

Phosphate ions were included at the nominal concentration of 4×10^{-2} M (~ 5 g KH_2PO_4 l^{-1}) though the concentration of phosphate ions in biological fluids is less than this, being between 1 to 6 mM depending on the fluid (blood) or tissue (liver) (Long 1961).

The temperature chosen in the programme was arbitarily selected as 25 °C as most stability constants are measured at this temperature. Increasing the temperature to 30 or 37 °C will not change the reported values significantly.

Results

Table 1 shows the Fe(III)-containing species taken into account in the speciation calculations.

For these speciation calculations, the equilibria shown in Table 2 were included.

Solubility of Fe(III)

The relationship between the solubility of Fe(III) with pH is shown in Figure 1. If an initial concentration of Fe higher than 10^{-6} M had been entered into the program this would have only affected the solubility of iron below pH 4 or above pH 12 where the available iron has reached complete solubilization. The common, but incorrect, statement that the concentration of soluble Fe(III) is only 10^{-18} M at room temperature (see for example: Neilands *et al.* 1987; Drechsel & Winkelmann 1997; Griffiths 1999) arises from a simplistic calculation based on the solubility product of $Fe(OH)_3$ of 10^{-39}. At pH 7 $[OH^-]$ is 10^{-7} M. This leaves $[Fe^{3+}]$ as $10^{-39}/10^{-21}$, i.e., 10^{-18} M. The speciation calculations show that at pH 7 in the absence of added ligands, the total concentration of soluble Fe(III) is approx. 1.4×10^{-9} M, with the main species in solution being $Fe(OH)_2^+$.

We would point out that, although this calculation for the solubility of Fe(III) gives a value some 10^9 greater than the value usually given, it is still nevertheless too low an effective concentration for Fe(III)

Table 1. Iron(III) species included in the calculations. Salicylic acid is written as H_2sal.

Iron(III) without ligand	Iron(III) salicylate	Iron(III) phosphate
Fe^{3+}(aq)	$Fe(sal)^+$	$Fe(H_2PO_4)^{2+}$
$Fe(OH)^{2+}$	$Fe(sal)_2^-$	$Fe(HPO_4)^+$
$Fe(OH)_2^+$	$Fe(sal)_3^{3-}$	$FePO_4$ solid
$Fe(OH)_3$ solid	$Fe(Hsal)^{2+}$	
$Fe(OH)_4^-$		

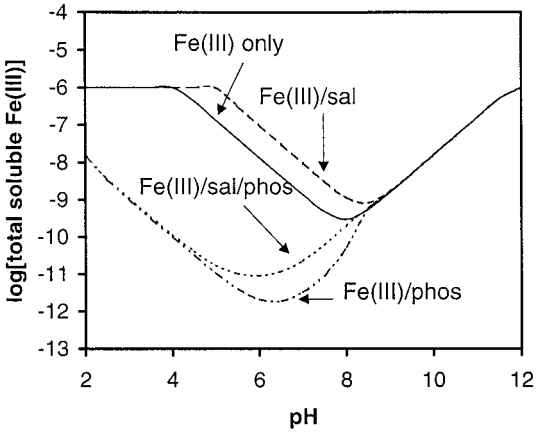

Figure 1. Concentration of total soluble iron(III) plotted against pH. Fe(III): 1×10^{-6} M iron(III) alone; Fe(III)/sal: 1×10^{-6} M iron(III) in presence of 1×10^{-4} M salicylic acid; Fe(III)/phos: 1×10^{-6} M iron(III) in presence of 4×10^{-2} M phosphate; Fe(III)/sal/phos: 1×10^{-6} M iron(III) in presence of 1×10^{-4} M salicylic acid and 4×10^{-2} M phosphate.

Table 2. Equilibria included in the calculations. Salicylic acid is written as H_2sal.

Equilibria involving iron(III)	Other equilibria
$Fe^{3+}(aq) + OH^- = Fe(OH)^{2+}$	$H_2sal = H^+ + Hsal^-$
$Fe^{3+}(aq) + 2OH^- = Fe(OH)^2_+$	$Hsal^- = H^+ + sal^{2-}$
$Fe^{3+}(aq) + 3OH^- = Fe(OH)_3$ (solid)	$H_3PO_4 = H^+ + H_2PO_4^-$
$Fe^{3+}(aq) + 4OH^- = Fe(OH)_4^-$	$H_2PO_4^- = H^+ + HPO_4^{2-}$
$Fe^{3+}(aq) + sal^{2-} = Fe(sal)^+$	$HPO_4^{2-} = H^+ + PO_4^{3-}$
$Fe^{3+}(aq) + 2sal^{2-} = Fe(sal)_2^-$	$H^+ + OH^- = H_2O$
$Fe^{3+}(aq) + 3sal^{2-} = Fe(sal)_3^{3-}$	
$Fe^{3+}(aq) + Hsal^- = Fe(Hsal)^{2+}$	
$Fe^{3+}(aq) + H_2PO_4^- = Fe(H_2PO_4)^{2+}$	
$Fe^{3+}(aq) + HPO_4^{2-} = Fe(HPO_4)^+$	
$Fe^{3+}(aq) + PO_4^- = FePO_4$ (solid)	

to be acquired directly from an environment. The production of a solubilizing agent, or siderophore, is therefore the major way in which iron will be made available to a microorganism.

Effect of salicylic acid

The effect of including salicylic acid into the calculations at a notional concentration of 10^{-4} M is also shown in Figure 1. The result was to increase the concentration of free Fe(III) at pH 7 to 9.8×10^{-9} M, that is by about 8.4×10^{-9} M from the concentration of Fe(III) in its absence. The proportions of the various species of iron from pH 2 to 12 in the presence of salicylic acid are shown in Figure 2. At pH 7, only about 2% of the iron is complexed with salicylate as the $Fe(sal)_2^-$ form; the remainder is $Fe(OH)_3$. Thus salicylate has a minimal effect on the solubilization of Fe(III).

Effect of phosphate

The effect of including phosphate into the calculations at 40 mM is also shown in Figure 1. As this causes the formation of ferric phosphate, the solubility of Fe(III) is decreased even further to 3.3×10^{-12} M.

Salicylic acid, also included into the calculations, has very little effect on the solubization of iron and its ineffectiveness in holding iron in solution is clearly demonstrated.

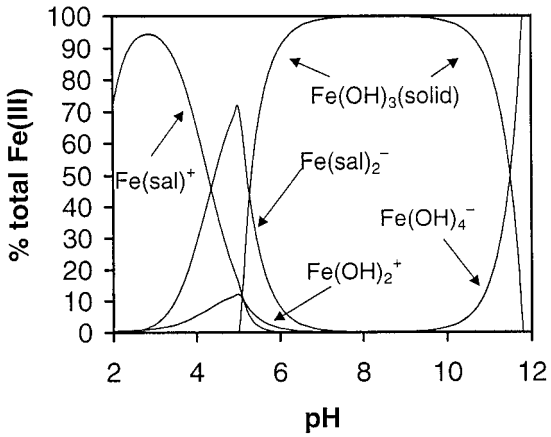

Figure 2. Speciation of iron(III) (total iron(III) 1×10^{-6} M) against pH in presence of 1×10^{-4} M salicylic acid.

Discussion

On the basis of these theoretical calcuations of the solublity of Fe(III) using data compiled from the major reference source on this topic (Martell & Smith 1977, 1982; Smith & Martell 1976, 1989) and now available via a database (Pettit & Powell 1999), there is no indication that an appreciable concentration of iron can be held in solution by salicylic acid. The concentration of iron in solution in the absence of salicylate is though much higher than previously indicated because these simple calculations had used only the solubility product of $Fe(OH)_3$ as their basis. The principle soluble form of iron that is present at pH 7, however, is $Fe(OH)_2^+$ and this gives a value of 1.4×10^{-9} M for the concentration of soluble iron.

These calculations effectively eliminate salicylate as a potential siderophore for Fe(III) both in the absence and presence of phosphate ions. Data for the related acid, 2,3-dihydroxybenzoic acid (DHB), which has also been suggested as a bacterial siderophore (Lopez Goni *et al.* 1992, 1995), is not available in the database and consequently similar calculations for its effect on iron solubilization cannot be carried out. Nevertheless from a theoretical standpoint, the two acids would be expected to behave similarly with respect to Fe(III) solubilization: DHB would be expected to chelate with Fe(III) between the carboxylic group and the 2-hydroxy group and thus be directly analagous to salicylate. On this basis we would therefore also reject the possiblity that this acid may also be siderophore. It too would have little effect on increasing the solubilization of Fe(III) at neutral pH values.

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