Supplemental Data

Structure, Dynamics, and Ionization Equilibria of the Tyrosine Residues in Bacillus circulans Xylanase

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<th>Tyrosine</th>
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<th>$^{1}\text{H} / ^{13}\text{C}\varepsilon$</th>
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*a* BcX in 10 mM sodium phosphate, pH 6.5, at 25 °C. Chemical shifts referenced directly to external DSS.

*b* Top (bottom) rows are for $\delta_1/\varepsilon_1$ (and $\delta_2/\varepsilon_2$).

*c* Could not be assigned reliably.
Fig. S1. Pulse sequence for a 1D $^{13}$C/$^{15}$N-filtered experiment to detect signals from $^1$H nuclei bonded directly to oxygen (or sulfur) atoms while suppressing those bonded directly to amide $^{15}$N or aromatic $^{13}$C nuclei (see Figures 1 and 4). Frequencies: $^1$H, 4.8 ppm (water); $^{13}$C, 126 ppm (aromatic region); $^{15}$N, 118 ppm (amide region). Delays: $\tau_a = 5.3$ ms and $\tau_b = 3.1$ ms. Hard 90° ($180^\circ$) pulses are depicted by tall narrow (wide) rectangles, and the 1.8 ms selective $^1$H 180° WATERGATE pulses as short rectangles. The triangles indicate 200 μs trapezoidal shape gradients at 46.9 G/cm ($g_1$). Although not required, $^{13}$C (2.36 kHz, WURST-40) and $^{15}$N (0.94 kHz, WALTZ-16) decoupling during acquisition improves filtering.

The phase cycling is:

$\phi_1 = 4(x, -x), 4(y, -y), 4(-x, x), 4(-y, y)$

$\phi_2 = 2(x), 2(y), 2(-x), 2(-y), 2(y), 2(-x), 2(-y), 2(x), 2(y), 2(-y), 2(x), 2(y), 2(-y), 2(x), 2(y), 2(-y), 2(x), 2(y), 2(-y), 2(x), 2(y), 2(-y), 2(x), 2(y), 2(-y), 2(x)$

$\phi_3 = 2(-x), 2(-y), 2(x), 2(y), 2(-x), 2(y), 2(-x), 2(y), 2(-x), 2(y), 2(-x), 2(y), 2(-x), 2(y), 2(-x), 2(y), 2(-x), 2(y), 2(-x), 2(y), 2(-x), 2(y), 2(-x), 2(y), 2(-x), 2(y), 2(-x), 2(y)$

receiver = 2(x, -x, -x, x), 2(y, -y, -y, y), 2(-x, x, x, -x), 2(-y, y, y, -y)
Fig. S2. Pulse sequence for a long-range 2D $^{13}$C-HSQC experiment to detect tyrosine $^{1}H^{\alpha} - ^{13}C^{\varepsilon}$ (and weaker $^{1}H^{\alpha} - ^{13}C^{\zeta}$) correlations and suppress direct $^{1}H - ^{13}C$ correlations (see Figures 1, 2 and 7). Frequencies: $^{1}H$, 4.8 ppm (water); $^{13}C$, 126 ppm (aromatic region). Spectral widths: $^{1}H$, 15000 Hz; $^{13}C$, 9654 Hz (96 complex $t_1$ increments) with a 600 MHz spectrometer. Delay $\tau_a = 9.4$ ms (or integer multiples of $1/(2*160$ Hz)). Hard 90° (180°) pulses are depicted by narrow (wide) rectangles. The triangles indicate trapezoidal shape z-gradients: $g_1 = 500$ µs, 1.8 G/cm; $g_2 = 300$ µs, -18.8 G/cm; $g_3 = 1.5$ ms, 28.0 G/cm; $g_4 = 600$ µs, -55.6 G/cm; and $g_5 = 200$ µs, 26.2 G/cm. Decoupling during acquisition (84 ms) is achieved with a 2.36 kHz WURST-40 field.

Pulses are applied along the x-axis except where indicated. The phase cycling is:

$\phi_1 = x, -x$ (plus States-TPPI incrementation for quadrature detection in $t_1$)
$\phi_2 = 2(x), 2(-x)$
$\phi_3 = 4(x), 4(-x)$
receiver = $x$, -$x$, -$x$, $x$
**Fig. S3.** Pulse sequence for a 2D $^{13}$C($^{13}$C-TOCSY)H$^a$ experiment to detect $^{13}$C$^a$-aromatic ring $^1$H correlations (adapted from (Löhr et al. 2005) and (Löhr et al. 2007)). Frequencies: $^1$H, 4.8 ppm (water); $^{13}$C, initially 35.5 ppm ($^{13}$C$^a$) then switched to 132.2 ppm (aromatic region). Spectral widths: $^1$H, 6993 Hz; $^{13}$C, 4223 Hz (35 complex $t_1$ increments) with a 600 MHz spectrometer. Delays: constant time $T_c = 8.5$ ms, $\tau = 3.4$ ms, $\delta = 10.4$ ms, and $\zeta = 14$ ms. Hard 90° (180°) pulses are depicted by narrow (wide) rectangles, and selective pulses as triangles or long filled bars. The tall filled bars are 500 µs 180° broadband WURST-10 pulses. The pulse labelled $\alpha$ is a 2.5 ms selective 180° I-BURP-2 at 58 ppm ($^{13}$C$^a$ region). The 2.7 ms 180° RE-BURP pulse in the middle of the $\zeta$ period is applied at 129 ppm. The two 180° phase modulated pulses on $^{13}$C$^a$ in the $\zeta$ period have the shape of the centre lobe of a sinc-function and a duration of 150 µs. The $^{13}$C-TOCSY consists of one cycle of a 3.3 kHz DIPS1-2. The $^{13}$C-$^1$H polarization transfer (CP) consists of one cycle of a 5.4 kHz DIPS1-2. The trapezoidal shape z-gradients have a duration of 500 µs and strengths: $g_1 = 7.5$ G/cm; $g_2 = 5.6$ G/cm; $g_3 = 14.0$ G/cm; $g_4 = 9.4$ G/cm; and $g_5 = 6.1$ G/cm. Decoupling during acquisition (88 ms) is achieved with a 2.36 kHz WURST-40 field.

Pulses are applied along x-axis except where indicated. The phase cycling is:

$\phi_1 = 4(x), 4(-x)$

$\phi_2 = 2(x), 2(-x)$ (plus States-TPPI incrementation for quadrature detection in $t_1$)

$\phi_3 = x, y$

$\phi_4 = 8(x), 8(-x)$

$\phi_5 = 16(x), 16(-x)$

$\phi_{adj}$ was adjusted to 84° for optimal signal

receiver = ($x, -x, -x, x), 2(-x,x,x,-x), (x, -x, -x, x)$
**Fig. S4.** Pulse sequence for the simultaneous measurement of tyrosine longitudinal $^{13}$C decay and conformational exchange rates (constant time version, adapted from (Farrow et al. 1994); see Figure 5). Frequencies: $^1$H, 4.8 ppm (water); $^{13}$C, 125 ppm (aromatic region). Spectral widths: $^1$H, 10000 Hz; $^{13}$C, 4080 Hz (64 complex $t_1$ increments) with a 600 MHz spectrometer. Delays: constant time $T_c = 15.8$ ms, $\tau = 2.5$ ms, and $\delta = 5$ ms. Mixing period delays $T$ are multiples of $N$ to give 10, 50, 100, 200, 300, 400, 600, 800 and 1000 ms. Hard 90° (180°) pulses are depicted by narrow (wide) rectangles. The triangles indicate trapezoidal shape $z$-gradients: $g_1 = 1$ ms, 9.4 G/cm; $g_2 = 0.5$ ms, 7.5 G/cm; $g_3 = 1$ ms, 28.1 G/cm; $g_4 = 1$ ms, -37.5 G/cm; $g_5 = 0.5$ ms, -43.1 G/cm; $g_6 = 0.5$ ms, 15.0 G/cm; $g_7 = 0.2$ ms, -46.8 G/cm. Decoupling during acquisition (64 ms) is achieved with a 1.74 kHz GARP-1 field.

Pulses are applied along x-axis except where indicated. The phase cycling is:

$\phi_1 = x$ (plus States-TPPI incrementation for quadrature detection in $t_1$)

$\phi_2 = x, y, -x, -y$

receiver = $x, -x$
Fig. S5. The pulse sequence for a two-dimensional $^{13}$C($^{13}$C')$^1$H$^\epsilon$ experiment to correlate tyrosine $^1$H$^\epsilon$ and $^{13}$C$^\zeta$ signals (see Figure 6C). The magnetization transfer follows an "out-and-back" pathway: $^1$H$^\epsilon$ $\rightarrow$ $^{13}$C$^\epsilon$ $\rightarrow$ $^{13}$C$^\zeta$(t$_1$) $\rightarrow$ $^{13}$C$^\zeta$ $\rightarrow$ $^1$H$^\epsilon$(t$_2$). Frequencies: $^1$H, 4.8 ppm (water); $^{13}$C, 118 ppm ($^{13}$C$^\epsilon$) and switched to 157.7 ppm ($^{13}$C$^\zeta$) during the t$_1$ period. Spectral widths: $^1$H, 8403 Hz; $^{13}$C, 754 Hz (40 complex t$_1$ increments) with a 600 MHz spectrometer. Delays: $\tau_a$ = 1.4 ms, $\tau_b$ = 1.35 ms, and $\tau_c$ = 3.45 ms (and the last delay includes half the duration of the 180° selective 2.9 ms RE-BURP pulse). Hard 90° (180°) pulses are depicted by narrow (wide) rectangles, and shaped pulses are triangles. The first two and last three carbon hard pulses are applied with full power ($B_1 = 15.8$ kHz), whereas the remaining carbon hard 90° pulses are selective ($B_1 = 1.546$ kHz). The first and last carbon shaped 180° pulses are selective 2.9 ms RE-BURP with maximum excitations at 118 ppm ($^{13}$C$^\epsilon$) and 157.7 ppm ($^{13}$C$^\zeta$). The 180° 144 µs rectangular pulse in the middle of the t$_1$ period has a maximum excitation at 118 ppm ($^{13}$C$^\zeta$). Shaped pulses were constructed with the Varian Pbox utility. The triangles indicate trapezoidal shaped z-gradients: $g_1$ = 500 µs, 8.1 G/cm; $g_2$ = 500 µs, 6.1 G/cm; $g_3$ = 3 ms, 15.2 G/cm; $g_4$ = 200 µs, 7.6 G/cm; $g_5$ = 750 µs, 5.6 G/cm; $g_6$ = 500 µs, 10.1 G/cm; $g_7$ = 200 µs, 6.6 G/cm; $g_8$ = 500 µs, 16.2 G/cm; $g_9$ = 500 µs, 60.8 G/cm. Decoupling during acquisition (86 ms) is achieved with a 2.36 kHz WALTZ-16 field.

Pulses are applied along x-axis except where indicated. The phase cycling is:

- $\phi_1 = 8(x)$, 8(-x)
- $\phi_2 = 2(x)$, 2(-x)
- $\phi_3 = x$, -x (plus States-TPPI incrementation for quadrature detection in t$_1$)
- $\phi_4 = 4(x)$, 4(-x)
- $\phi_5 = 16(x)$, 16(-x)

receiver = x, 2(-x), x, -x, 2(x), 2(-x), 2(x), -x, x, 2(-x), x.
**Fig. S6.** The pulse sequence for a three-dimensional $^{13}$C $^{13}$C $^{1}$H $^e$ experiment to correlate tyrosine $^{1}$H $^e$, $^{13}$C $^r$ and $^{13}$C $^z$ signals (see Figure 6D). The magnetization transfer pathway is $^{1}$H $^e$ $\rightarrow$ $^{13}$C $^r(t_2)$ $\rightarrow$ $^{13}$C $^z(t_1)$ $\rightarrow$ $^{13}$C $^r$ $\rightarrow$ $^{1}$H $^e(t_3)$. Due to the non-selective character of the $180^\circ$ $^{13}$C pulse applied inside the $2^*\tau_g$ delay for constant time $t_2$ evolution, only half of the magnetization originating from $^{1}$H $^e$ is returned. All parameters are the same as for the two-dimensional $^{13}$C $^z$($^{13}$C $^r$) $^{1}$H $^e$ (Figure S5) with the following exceptions: additional delays $\tau_f = 2^*\tau_b$ and $\tau_g = 19$ ms; $^{13}$C $^r$ spectral width 903 Hz, 25 complex $t_1$ increments, and 17 complex $t_2$ increments; $^{1}$H decoupling (8.3 kHz) and $^{13}$C decoupling (2.2 kHz) are accomplished with WALTZ-16 modulation.

Quadrature detection in $t_1$ and $t_2$ is obtained by States-TPPI incrementation of $\phi_3$ and $\phi_2$, respectively.
**Fig. S7.** The pulse sequence for a two-dimensional \(^{13}\text{C}^{\varepsilon - \delta}\)^{1}H^{\varepsilon - \delta} experiment to correlate tyrosine \(^1\text{H}^{\varepsilon - \delta}\) and \(^{13}\text{C}^{\varepsilon}\) signals (see Figure 6A). The magnetization transfer pathway is:

\[
\begin{align*}
\text{\(^1\text{H}^{\varepsilon}\)} (t_2) & \\
\text{\(^1\text{H}^{\varepsilon}\)} & \quad \rightarrow \quad \text{\(^{13}\text{C}^{\varepsilon}\)} \quad \rightarrow \quad \text{\(^{13}\text{C}^{\varepsilon}\)} (t_1) \quad \rightarrow \quad \text{\(^{13}\text{C}^{\delta}\)} \quad \rightarrow \quad \text{\(^1\text{H}^{\delta}\)} (t_2)
\end{align*}
\]

All parameters are the same as for the two-dimensional \(^{13}\text{C}^{\varepsilon - \varepsilon}\)^{1}H^{\varepsilon - \varepsilon} (Figure S5) with the following exceptions: delays \(2*\tau_d = 5.6\) msec and \(2*\tau_e = 4\) ms (the last delay includes the duration of the \(180^\circ\) \(^{13}\text{C}\) selective \(490\) \(\mu\)s R-SNOB pulse); \(^1\text{H}^{\varepsilon - \delta}\) spectral width: \(8403.4\) Hz; \(^{13}\text{C}\) carrier set to \(118\) ppm \((^{13}\text{C}^{\varepsilon})\), switched to \(157.7\) ppm \((^{13}\text{C}^{\varepsilon})\) immediately before and after the \(t_1\) evolution period (50 complex \(t_1\) increments), and then switched to \(126\) ppm (midway between \(^{13}\text{C}^{\varepsilon}\) and \(^{13}\text{C}^{\delta}\)) for the remaining \(^{13}\text{C}\) pulses. The ratio of detected \(^1\text{H}^{\varepsilon}\) and \(^1\text{H}^{\delta}\) intensities depends on \(\tau_d\). The duration and strength of the \(g_{10}\) gradient are \(500\) \(\mu\)s, \(4\) G/cm.

Quadrature detection in \(t_1\) is obtained by States-TPPI incrementation of \(\phi_3\).
**Fig. S8.** The pulse sequence for a three-dimensional $^{13}$C$^{13}$C$^{13}$C$\delta$ experiment to correlate tyrosine $^1$H$\delta$, $^{13}$C$\epsilon$ and $^{13}$C$\zeta$ signals (see Figure 6B). The magnetization transfer pathway is:

$$^1$$H$^\prime$ (t$_3$)

$$^1$$H$^\prime$ $\rightarrow$ $^{13}$C$\epsilon$ (t$_2$) $\rightarrow$ $^{13}$C$\zeta$ (t$_1$) $\rightarrow$ $^{13}$C$\epsilon$ $\rightarrow$ $^{13}$C$\delta$ $\rightarrow$ $^1$H$^\delta$ (t$_3$)

All parameters are the same as for the two-dimensional $^{13}$C$\zeta$($^{13}$C$\epsilon$)$^1$H$\delta$ (Figure S7) with the following exceptions: additional delays $\tau_f = 2\tau_b$ and $\tau_g = 19$ ms; $^{13}$C$\epsilon$ spectral width of 905 Hz (25 complex t$_1$ increments, and 17 complex t$_2$ increments) using a 600 MHz spectrometer.

Quadrature detection in t$_1$ and t$_2$ is obtained by States-TPPI incrementation of $\phi_3$ and $\phi_2$, respectively.
**Fig. S9.** The $^{13}$C and $^1$H chemical shifts (ppm) of free tyrosine at pH 3.6 and 12.5, measured from $^{13}$C-HMBC spectra (10 mM sodium phosphate, 5% D$_2$O, 25 °C). These values agree with those reported for glycyl-L-tyrosine amide and glycyl-L-tyrosineglycine (Norton and Bradbury 1974), as well as tyrosine residues in a linear tetrapeptide Gly-Gly-Tyr-Ala (Richarz and Wüthrich 1978; Bundi and Wüthrich 1979). However, one exception is that a larger pH-dependent $^{13}$C$\gamma$ chemical shift change ($|\Delta \delta|$ = 6.2 ppm or 6.8 ppm) is observed in the latter species, respectively, compared to the free amino acid.
**Fig. S10.** The pH dependent $^1\text{H}^\delta$ (left) and $^1\text{H}^\varepsilon$ (right) chemical shifts of the BcX tyrosines (35 °C), measured from $^1\text{H}^\varepsilon/^1\text{H}^\delta$ ($^{13}\text{C}^\varepsilon$)$^{13}\text{C}^\zeta$ spectra. The lines represent the best-fits to a single titration event. In contrast to $^{13}\text{C}^\zeta$ (Figure 7), the $^1\text{H}^\delta$ and $^1\text{H}^\varepsilon$ nuclei show significantly more dispersion due to their structural environment than any changes due to the effects of pH, and thus their chemical shifts are not diagnostic of the tyrosine ionization states. Fitting the data for residues showing clear, albeit small, pH-dependent changes ($|\delta_b - \delta_a| > 0.1$ ppm) yielded the following apparent p$K_a$ values:

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<th>p$K_a$ (from $^1\text{H}^\varepsilon$)</th>
<th>$^1\text{H}^\varepsilon$ δ (ppm)</th>
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<td>6.8 ± 0.1</td>
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<td>Y88</td>
<td>8.9 ± 0.2</td>
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<td>Y94</td>
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<td>Y113</td>
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Note that the fit p$K_a$ values for Tyr113 and Tyr174 are similar for both the $^1\text{H}^\delta$ and $^1\text{H}^\varepsilon$, and also agree with those determined from their pH-dependent $^{13}\text{C}^\zeta$ shifts (Table 1). In contrast, whereas the $^1\text{H}^\varepsilon$ and $^{13}\text{C}^\zeta$ of Tyr88 yield comparable p$K_a$ values, the $^1\text{H}^\delta$ appears to report the titration of other group(s) in BcX with a p$K_a$ ~8.9; however, no obvious candidate can be identified from the crystal structure of the enzyme. The substantial change in the $^1\text{H}^\delta$ chemical shift of Tyr69 most likely reflects the ionization of the neighbouring catalytic general acid Glu172 (p$K_a$ 6.7). (McIntosh et al. 1996; McIntosh et al. 2011)).
Supplemental References


